OPTIMIZATION AND UTILIZATION OF IMMATURE SPIKE CULTURE SYSTEM TO IDENTIFY AND CHARACTERIZE FUSARIUM HEAD BLIGHT RESISTANT WHEAT GENOTYPES

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ABSTRACT

Fusarium head blight (FHB) is a potentially devastating fungal disease-causing yield loss and grain quality reduction in wheat, barley, oat and other small grain cereals. In wheat (*Triticum aestivum* L.) FHB is predominantly caused by *Fusarium graminearum* Schwabe [telomorph: *Gibberella zeae* (Schaw) Petch), although a few other species also cause FHB. Fusarium infects wheat immature spikes during flowering and produces a trichotehecene group of mycotoxins that are toxic to humans and animals, but also act as virulence factors responsible for the spread of the disease in the plant tissue. To mitigate the effect of FHB, there is a need to develop durable FHB resistance in wheat. To select for durable FHB resistance, an immature spike culture-based method was developed to distinguish FHB resistant and susceptible genotypes. Screening of an ethyl methyl sulfonate (EMS) treated spike culture derived variant (SCDV) population (134 lines) of wheat cultivar AC Nanda identified 15 FHB-resistant and 9 FHB-susceptible lines.

A LC-MS/MS method was optimized to quantify type B trichothecenes in infected wheat spikes. The optimized method separated four mycotoxins: DON, 15-ADON, 3-ADON and Nivelanol; and DON-3-Glycoside (D3G) within 25 min with intermediate precision and repeatability. The coefficient of determination was >0.99 for all the mycotoxins whereas, the level of detection and quantification ranged from 0.30 to 0.75 and from 0.5 to 2.5 ng/mL, respectively. The FHB resistant genotypes had reduced concentrations of all mycotoxins compared to the FHB-suscpeptible genotypes, suggesting the utility of the method to determine grain mycotoxin concentration and to identify the chemotype of a *F. graminearum* isolate.

UDP-glucosyl transferases (*UGT*) catalyze the conversion of DON to non-toxic D3G by transferring a glucoside moiety to the 3'- carbon on DON which contributes to FHB resistance, thus preventing the spread of the fungus. Nucleotide sequence comparisons revealed one nucleotide change in *TaUGT-2B* and *TaUGT-3B* between AC Nanda and Sumai-3. Statistical analysis determined that the nucleotide difference present at positions 450 and 1558 bp from the translation initiation site between AC Nanda and Sumai-3 in the *TaUGT-2B* and *TaUGT-3B* genes respectively correlated significantly ($P<0.01$) with the disease severity of 134 SCDV lines.

FHB- resistant and susceptible lines were also screened in FHB nurseries at

Carman Manitoba (University of Manitoba) (2017) and University of Saskatchewan (2017, 2018). FHB disease development and severity, observed during *in vitro* spike culture screening method, showed a positive correlation with mycotoxin accumulation in the spikes as determined using a LC-MS/MS method, and FHB disease development and severity in the field experiments. Theses results supported the utilization of *in vitro* spike culture to identify FHB resistant SCDV lines that can potentially be used in breeding programs to develop FHB resistant wheat germplasm.

The immature spike cultures from 55 SCDV lines were separately inoculated with five *F. graminearum* isolates, Carman-NIV (NIV), Carman-705-2 (3-ADON), M9-07-1 (3-ADON), M1-07-2 (15-ADON), and China-Fg809 (15-ADON) that produced different mycotoxins (in parenthesis). Five genotypes with stable FHB resistance were identified based on their FHB disease development and severity at 5, 7, 9, and 11 days after inoculation (DAI). Compared with the susceptible control AC Nanda, all the resistant genotypes had significantly reduced FHB susceptibility. LC-MS/MS analysis identified five DON chemotypes. The SCDV lines FHB213.4, FHB244.1, FHB245.6, FHB250.2, and FHB252.3 were identified as having resistance to multiple *F. graminearum* chemotypes.

The SCDV lines FHB202, FHB244, FHB250 and FHB256 were also used as resistant parent to cross with an elite wheat cultivar PT588 (CDC Hughes) to generate FHB resistant wheat breeding populations. The genotypes with the same SNP as Sumai-3 in crossing combinations FHB202/PT588, FHB244/PT588, FHB250/PT588 and FHB256/PT588 accounted for 5.88%, 9.09%, 34.62% and 10.53% within each BC_2 progeny population, with an average rate of 15.03%. The rates of FHB resistance in crossing combinations of PT588*2/FHB202, PT588*2/FHB244, PT588*2/FHB250 and PT588*2/FHB256 were 0.0%, 0.0%, 17.39% and 27.27%, with an average of 11.17%. The ANOVA results showed significant differences of phenotypic FHB severity among different genotypic groups ($P<0.01$), indicating a strong correlation between phenotypic and genotypic data.

In conclusion, the study demonstrated the use of immature spike cultures as a method to screen for FHB disease development and resistance. A LC-MS/MS method was optimized to characterize four mycotoxins and D3G commonly present during FHB infection. The SCDV population yielded FHB resistant lines, which were used to cross with CDC Hughes to develop FHB resistant wheat genotypes.

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LIST OF ABBREVIATIONS

3ADON	:	3-Acetyl-Deoxynivalenol
15ADON	:	15-Acetyl-Deoxynivalenol
ANOVA	:	Analysis of Variance
BC_2	:	The second generation of Back Cross
CAD	:	Collisionally Activated Dissociation
CAPS	:	Cleaved Amplified Polymorphic Sequence
cDNA	:	Complementary DNA
CE	:	Collision Energy
CRD	:	Complete Randomized Design
CUR	:	Curtain Gas
CXP	:	Collision Exit Potential
D3G	:	Deoxynivalenol-3-Glucoside
DAD	:	Diodearray
DAI	:	Days After Inoculation
DNA	:	Deoxyribonucleic Acid
DON	:	Deoxynivalenol
DP	:	Declustering Potential
ECD	:	Electron Capture
EIN2	:	Ethylene Insensitive Gene-2
ELISA	:	Enzyme-Linked Immuno Sorbent Assay
EMS	:	Ethyl Methane Sulfonate
EP	:	Entrance Potential
EPT	:	12, 13-epoxytrichothec-9-ene
ESI	:	Electrospray Ionization
EST	:	Expressed Sequence Tag
FD	:	Fluorescence Detection
FDK	:	Fusarium Damaged Kernel
FGSC	:	Fusarium Graminearum Species Complex
FHB	:	Fusarium Head Blight
FID	:	Flame Ionization Detector
GBS	:	Genotyping by Sequencing

GC	:	Gas Chromatography
GCPSR	:	Genealogical Concordance Phylogenetic Species Recognition
GS1	:	Nebulizer Gas
GS2	:	Heater Gas
HPLC	:	High Performance Liquid Chromatography
HRM	:	High Resolution Melt
INC	:	Incidence
IS	:	Ion Voltage Spray
LC-MS/MS	:	Liquid Chromatography-Tandem Mass Spectrometry
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
MIP	:	Molecular Imprinted Polymer
mRNA	:	Messenger Ribonucleic acid
MS	:	Mass Spectrometry
MSS	:	Mean Sum of Squares
NGS	:	Next Generation Sequencing
NIV	:	Nivalenol
PCR	:	Polymerase Chain Reaction
PSS	:	Percentage of Symptomatic Spikelets in a Spike
QTL	:	Quantitative trait locus
RE	:	Recovery Rate
RNA	:	Ribonucleic Acid
RSD	:	Relative Standard Deviation
SCDV	:	Spike Culture Derived Variant
SEV	:	Severity
SNP	:	Single Nucleotide Polymorphism
SPR	:	Surface Plasmon Resonance
SSE	:	Matrix induced signal suppression or enhancement
SSRs	:	Simple Sequence Repeats
TEM	:	Turbo Ion Spray Interface Temperature
TILLING	:	Targeting Induced Local Lesions In Genomes
TLC	:	Thin-layer Chromatography
UGT	:	UDP-glucosyl transferases

UV	:	Ultraviolet
VRI	:	Visual Rating Index

CHAPTER 1. INTRODUCTION

1.1 Backgound

Wheat (*Triticum aestivum* L.) production is approximately 740 metric tonnes (Mt) annually, which is similar to rice, and only exceeded by maize at approximately 1 B Mt. Rice is limited to the tropical or sub-tropical regions of the world, while wheat is cultivated in the most diverse agroclimatic regions from the tropics to the edges of Arctic Circle (Worland and Snape, 2001). Wheat is the staple food of 40% of the human population worldwide (Shewry and Hey, 2015), providing 21% of the calories and 20% of the protein for diets in developing countries (Pena-Bautista et al., 2017).

The availability of adequate amounts of wheat for human dietary needs is of major importance for food security in many parts of the world. Crop improvement to increase wheat production has been a major challenge to meet the dietary needs of a growing human population and reduction in arable land due to the anthropogenic activities. Plant breeding has consistently increased wheat yields from 0.3 to 0.7% per year from the unfavorable to most favorable wheat growing regions (Hucl et al., 2015). The increased anthropogenic activities also cause climate change manifest as higher temperatures, erratic precipitation, ozone layer damage and increased atmospheric carbon dioxide concentrations (Knox et al., 2016). The effects of climate change result in extreme and altered weather patterns that cause abiotic and biotic stresses for crops. One biotic stress is the plant pathogens that cause diseases of crops (Abberton et al., 2015; Rasheed et al., 2017), resulting in yield losses. Among the major biotic stresses of wheat worldwide, Fusarium head blight (FHB) is the most damaging and hazardous wheat floral or spike disease. Every four or five years, serious FHB epidemics have occurred in various countries (Figueroa et al., 2018).

Fusarium head blight, or scab, is a serious fungal disease caused by *Fusarium graminearum* in wheat and small grain cereals as well as ear/stalk rot in maize (Zhou et al., 2002). It produces ascospores (sexual spores) or conidia (asexual spore) that are resistant to environmental stress and thus survive over winter on or within seed and crop residue (Geng et al., 2014). Infected crop residue acts as the primary inoculum source for *F. graminearum* (Keller et al., 2014). Upon exposure to favorable conditions, the spores are wind blown or rain splashed to the wheat spike at anthesis, germinate and infect at temperatures of 25-28^{o}C and precipitation and high humidity for at least 12

hours (Bai and Shaner, 1994; McMullen et al., 1997; Zhou et al., 2002; Simsek et al., 2012). FHB induces premature bleaching of spikes resulting in sterility or under-developed kernels called Fusarium-damaged kernels (FDKs) or tombstone kernels (Wegulo, 2012), thus substantially affecting grain yield. FHB also contaminates wheat kernels with mycotoxins affecting processing technology (like fermentation) and end user health (Champeil et al., 2004).

The *Fusarium graminearum* species complex produces deoxynivalenol (DON) and its derivatives 3-ADON and 15-ADON, as well as nivalenol (NIV). Based on the mycotoxins produced by *F. graminearum*, the isolates are grouped into the following chemotypes: NIV, 3-ADON and 15-ADON (Foroud et al., 2012). The 3-ADON and 15-ADON chemotypes produce DON (McMullen et al., 2012). NIV is more toxic to livestock compared to DON, but it is not common in Canadian wheat (Tittlemier et al., 2013). Until recently the 15-ADON chemotypes were predominant in Canada, but at present 3-ADON chemotypes occur more frequently (Gilbert and Haber, 2013). DON is considered a virulence factor in FHB; it is critical for symptom development after infection. DON also plays an important role in the pathogen's growth and survival under varying environmental conditions (Audenaert et al., 2013). Not a single isolate of *F. graminearum* has been identified to produce both NIV and DON (Goswami and Kistler, 2005). In the Red River Valley of Manitoba, the 3-ADON chemotype is more prevalent than the 15-ADON chemotype. This trend is similar to that reported across North America, where there is a shift from 15-ADON to 3-ADON chemotypes (Ward et al., 2008). This shift may affect the chemical control of FHB and pose a challenge to disease control (Ward et al., 2008; Amarasinghe et al., 2013).

Increasing FHB problems over the years in Canada is a matter of great concern to wheat producers, scientists and industry. Integrated disease management practices include the cultivation of resistant wheat cultivars and diverse crop rotations, in combination with the application of fungicides, providing reinforced protection from pathogen infection and mycotoxin production (Edwards, 2004; Del Ponte et al., 2012). Utilizing FHB resistant cultivars in an integrated disease management program is one of the most effective strategies to control FHB and minimize the trichothecene toxin contamination in both processed and unprocessed grain products (Beres et al., 2018).

Improving crop management practices and strengthening host resistance are two major strategies to control FHB in wheat (Beres et al., 2018). There is no single strategy

that can control FHB, therefore, an integrated approach is needed to reduce losses. Crop management practices, such as tillage, crop rotation and use of fungicides and bio-control products are recommended (Wegulo et al., 2015); however, such practices are environmentally sensitive and also increase input costs to the producer. Therefore, developing FHB resistant cultivars is a more desirable strategy to reduce FHB infection. FHB resistance in wheat is a quantitative trait governed by several genes (Buerstmayr et al., 2009). Some resistant genotypes like Sumai-3 and Nobeokabouzu have been reported but they are not adapted to Canadian environments and have poor agronomic performance. Consequently, durable FHB resistance in wheat has not been achieved to-date. The availability of a reliable and rapid method to identify FHB resistant genotypes is needed to make progress towards developing FHB resistant genotypes.

1.2 Hypotheses

1. *In vitro* immature spike culture can be used to screen for FHB resistance.
2. In *in vitro* immature spikes, mycotoxin production in grain is correlated with FHB severity.
3. DNA based molecular markers can be used to follow FHB resistance.

1.3 Objectives

The main objectives of the work reported in this thesis were as follows:

1. Utilize the recently optimized *in vitro* spike culture method (Ganeshan et al., 2010) to develop an FHB screening method;
2. Identify and characterize FHB resistant genotypes from an ethyl methyl sulfonate (EMS) mutagenized spike cultured derived variant (SCDV) population (Ganeshan and Chibbar, 2017);
3. Develop DNA markers associated with FHB resistance and use them to study the introgression of FHB resistance in the progeny of crosses made between FHB resistant genotypes and elite wheat breeding lines / cultivars.

CHAPTER 2. LITERATURE REVIEW

2.1 Wheat production and contribution to human diet

Wheat (*Triticum aestivum* L.) is one of the top three crops cultivated world-wide. During 2015-2016, wheat production increased from 737 Mt to 749 Mt, making it second to maize (1,010-1,060 Mt), and similar to rice (740 ~ 741 Mt) (FAO Stat, 2016). Canada is among the top five wheat producing countries in the world with a total production of 30.5 Mt (FAO Stat, 2016). In Canada, the three Prairie Provinces of Alberta, Saskatchewan and Manitoba are the major contributors to field crop production, including 90% of the wheat production in Canada (Stats Canada, 2017).

Wheat is a very versatile cereal grain that is used to prepare diverse foods that are regional and ethnically specific. In human diet, wheat contributes one-fifth of the total dietary calories (carbohydrates) and proteins, making it an essential component of food security (Shiferaw et al., 2013). It is also a good source of dietary fiber, and minor components such as lipids, vitamins, minerals and bioactive phytochemicals needed for a healthy diet (Shewry and Hey, 2015).

2.1.1 Wheat

Common wheat (*Triticum aestivum* L.) is an allohexaploid (2n = 6x = 42, AABBDD), with a genome size of 16,700 Mb/1C (Bennett et al., 2000). The donors of the A, B and D genomes are *Triticum monococcum* L. (including var. *boeoticum* and var. *urartu*), *Aegilops speltoides* Tausch and *Aegilops squarros*a L. (syn. *Triticum tauschii*). The introduction of D genome broadened the adaptability of wheat, making it a primary crop cultivated in diverse regions of the world. Currently wheat is cultivated from 67^{o}N in Scandinavia and Russia to 45^{o}S in Argentina, including elevated regions in the topics and sub-tropics (Feldman, 1995; Shewry and Hey, 2015). Hexaploid wheat is less variable than its diploid progenitors, suggesting a genetic bottleneck due to few initial hybridizations that gave rise to modern day wheat (Appels and Lagudah, 1990). An effective method to improve yield, quality, and resistance to biotic and abiotic stresses of wheat is to broaden the genetic base of cultivated wheat (Trethowan and Mujeeb-Kazi, 2008; Tester and Langridge, 2010). The primary gene pool includes hexaploid landraces, cultivated tetraploid *Triticum turgidum* (2n = 4x = 28, AABB) and its wild form *Triticum dicoccoides*, *Triticum monococcum* (including var. *boeoticum*

and var. *urartu*), and *Aegilops squarrosa* (syn. *Triticum tauschii*). There is much variation in the wild relatives of wheat, which contain abundant genetic resources that could be used as donors to broaden the genetic base of wheat (Pingali and Rajaram, 1999; Friebe et al., 1996; Tester and Langridge, 2010). The secondary gene pool is comprised of closely related, mostly polyploid *Triticum* and *Aegilops* species that share one common genome with wheat. The tertiary gene pool is a composite of diploid and polyploid species containing genomes that are nonhomologous to those of wheat.

2.1.2 Climate change and its influence on wheat production

The world's population is growing rapidly, and is predicted to reach 9 billion by 2050. Increased anthropogenic activity has changed surface solar radiation, increased atmospheric carbon dioxide concentration and temperature, and caused erratic precipitation patterns. All these factors affect crop development and production and pose a major threat to agriculture. The edaphoclimatic conditions that cause abiotic stress can be mitigated by the use of adapted cultivars or agronomic management practices (Challinor et al., 2014; Moore and Lobell, 2014). Breeding climate-resilient crops is a major challenge, but it has the potential to mitigate the adverse effects of climate change.

2.1.3 Biotic factors and fungal diseases

Climate change induces abiotic stresses and it also affects the habitat of pathogens, facilitatin their spread (Bale et al., 2002; Luck et al., 2011; Madgwick et al., 2011; Nicol et al., 2011). A plant disease is the result of an interaction of a susceptible host plant with a virulent pathogen in an environment conducive to the proliferation of invading pathogen. Several factors play a role, but environmental conditions such as temperature, humidity, and increased ozone and carbon dioxide concentrations play important role in pathogen infection and disease spread. Fungal diseases have been estimated to cause 15-20% wheat yield losses annually (Figueroa et al., 2018). It has been predicted that climate change induced temperature increase and erratic precipitation pattern will cause several pathogens to spread into new geographic areas (Elad and Pertot, 2014). Wheat is inflicted with several diseases such as rusts, leaf spots, Fusarium head blight and blast (Figueroa et al., 2018). Among wheat diseases, Fusarium head blight (FHB) or scab caused by *Fusarium graminearum* is globally one

of the most serious diseases whose recent spread has been influenced by climate change and conservation farming practices such as no-till (Chakraborty and Newton, 2011). The first reported FHB epidemic was in 1884 in England where it was called 'wheat scab' (Dubin et al., 1997). Since then the disease has spread world-wide, and sporadic epidemics have been reported (Dubin et al., 1997; Bai and Shaner, 2004; Buerstmayer et al., 2009; McMullen et al., 2012). In Canada prior to 1980, FHB epidemics were uncommon, but the frequency has increased during the last quarter of twentieth century (Beres et al., 2018). During the 1980s and 1990s, FHB sometimes occurred in Ontario and Manitoba where warm temperatures, high humidity and precipitation during wheat anthesis resulted in DON levels of 1 ppm or more in the harvested grain (Hooker et al., 2002). Epidemics of FHB have moved westward into Saskatchewan and Alberta since the dry conditions of 2001 – 2005 have subsided (Beres et al., 2018). An FHB survey of 89 Saskatchewan winter wheat crops in 2013 identified 25 out of 89 (28%) infected by FHB. The mean values of incidence, severity and FHB indices were 4.6, 11 and 0.7%, respectively. The FHB incidence varied from 0.5-11.0%, severity from 0.8-62.5%, and FHB index from <0.01 to 4.69% (Brar et al., 2014).

2.2 Fusarium Head Blight (FHB)

Fusarium head blight is an extremely detrimental disease of small grain cereals, including wheat, barley, oats, rye, and triticale, as well as maize, in humid and temperate regions (Wegulo et al., 2015). In wheat, FHB is caused by several Fusarium species, which include *F. graminearum*, *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. poae*, *F. verticilloides*, *F. pseudograminearum*, and *F. sprotrichoides* (Parry et al., 1995); however, *F. graminearum* is the main causal organism for FHB around the world. Fusarium infection produces DON, which is a virulence factor that inhibits host protein synthesis, resulting in necrosis, chlorosis, and wilting of plants (McLaughlin et al., 1977; Lemmens et al., 2005). FHB causes severe economic losses by reducing grain yield and quality, downgrading grain and loss of market value. The yield loss is due to flower sterility resulting in shriveled kernels with reduced protein content and contamination with the mycotoxins, especially deoxynivalenol (DON) (Wong et al., 1995; Wu et al., 1996, 1997). DON contaminated seeds are detrimental to human and animal health (Bai et al., 2001; Zhou et al., 2002).

2.2.1 The Fusarium species complex (taxonomy of the species)

The Fusarium genus belongs to the phylum Ascomycota, class Ascomycetes, and order Hypocreales. The teleomorph of *Fusarium* spp. is the genus *Gibberella* (Ma et al., 2013). The initial characterization of *Fusarium* spp. was based on major morphological traits such as the size and shape of spores, macroconidia, microconidia, and chlamydospores; and conidiogenous cells (Wollenweber and Reinking, 1935; Leslie and Summerell, 2006). A major limitation of the morphological traits to distinguish species is that in micro-fungi the number of readily distinct morphological features is less than the number of species that need to be classified. The biological species concept (Mayr, 1940; 1963) is based on populations that have the potential to mate or interbreed with each other. The major challenge to apply the biological species concept to *Fusarium* spp. is that several are asexual and rarely or never produce a sexual stage (Nelson, 1991; Summerell et al., 2010). The phylogenetic species concept determines the quantitative difference of genetic relatedness based on DNA sequencing of selected genes (Taylor et al., 2000). Characterization of the phylogeny of multiple genetic loci combined with the genealogical concordance of phylogenetic species recognition (GCPSR) identified 16 monophyletic species of *F. graminearum* and resulted in renaming "*F. graminearum*" with "*F. graminearum* species complex (FGSC)" (O'Donnell et al., 2004; Starkey et al., 2007; Sarver et al., 2011; Amarasinghe, 2016). These 16 species include: *F. austroamericanum*, *F. acacia-mearnsii*, *F. asiaticum*, *F. aethiopicum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae*, *F. graminearum* sensu stricto, *F. gerlachii*, *F. meridionale*, *F. mesoamericanum*, *F. louisianense*, *F. nepalenses*, *F. vorosii*, *F. ussurianum*, and a genetically distinct U.S. Gulf Coast population of *F. graminearum* s.s. (O'Donnell et al., 2004; Starkey et al., 2007; Sarver et al., 2011; Amarasinghe, 2016). The *F. graminearum* (teleomorph, *Gibberella zeae*) species complex (FGSC) is the predominant FHB causal pathogen of wheat in Manitoba, eastern Saskatchewan, eastern Canada, and in the United Sates (Gilbert and Tekauz, 2000; Cuthbert et al., 2006).

Mycotoxin contamination of Fusarium infected cereals is one of the most problematic results of FHB. There are more than 140 mycotoxins produced by *Fusarium* spp., including trichothecenes, zearalenone, fumonisins and moniliformin (Sobrova et al., 2010; Desjardins et al., 1993; Foroud and Eudes, 2009; Wang et al., 2011).

2.2.2 Life cycle, disease process, and symptoms of Fusarium head blight

Fusarium graminearum is a hemibiotrophic fungus. FHB is a monocyclic disease because after primary infection no, or very little little secondary infection occurs. Therefore, it has only one infection cycle during a growing season (Bai and Shaner, 1994; Shaner, 2003).

2.2.2.1 Life cycle of Fusarium

The development of disease is determined by the interaction of the factors in the disease triangle: the pathogen, the host, and the environment (Shaner, 2003). Infection occurs upon interaction of these three factor to initiate the disease cycle (Figure 2.1). FHB epidemics are due to the built up of natural inoculum caused by the changing patterns of tillage and crop rotations (Dill-Macky and Jones, 2000; McMullen et al., 1997) combined with the favorable environmental factors (McMullen et al., 2012). In the field, the primary source of inoculum is the ascospores and conidia that remain in the crop residue of wheat and corn, upon which the pathogen grows (Hilton et al., 1999; Yan et al., 2011). During the growing season, warm, windy, and humid weather favors the production of fruiting structures, the sporodochia, which bear the asexual macroconidia, followed by development of perithecia during the sexual stage resulting in the production of ascospores. Macroconidia and ascospores are the principal inoculum sources during FHB epidemics, although other sources of inoculum also exist such as chlamydospores and hyphal fragments (Sutton, 1982; McMullen et al., 1997; Fernando et al., 2000; Inch and Gilbert, 2003; Liddell, 2003; Osborne and Stein, 2007). The primary inoculum, the ascospores, spread to wheat spikes by rain-splash or wind (McMullen et al., 2012), followed by initiation of infection on susceptible hosts (Fernando et al., 1997; Shaner, 2003). The infection process is favored by prolonged warm, wet and humid weather during anthesis (Li and Yen, 2008) and continues to the soft dough stage (McMullen et al., 2012). The optimum temperature for the production of F. graminearum perithecia is 29℃, and 25-28℃ for ascospore formation. The release of ascospores occurs between 10-30℃, optimum 16℃ (epidemiological reference needed here. Above 26℃, no ascospore discharge occurs (Sutton, 1982). At 90 to 100% relative humidity, germination of ascospores begins within 4 hours at 20℃ (Beyer and Verreet, 2005), and do not germinate below 50% relative humidity (Beyer and Verreet, 2005; Gilbert et al., 2008). The premature bleaching of the spikes is a typical symptom

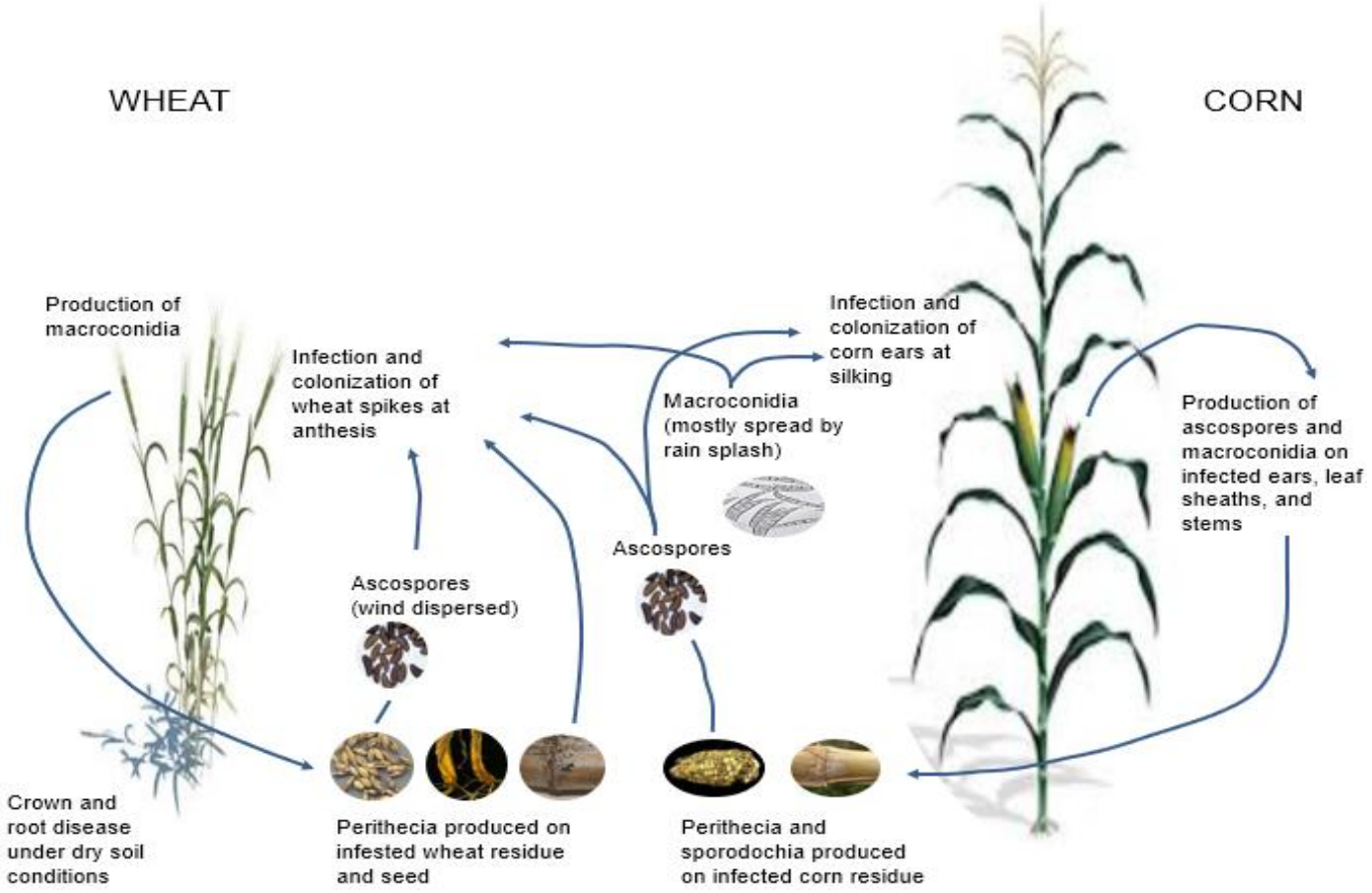


Figure 2.1: Wheat fusarium head blight (FHB) disease cycle. (Modified from Bailey et al., 2003).

of FHB.

2.2.2.2 Fusarium head blight development

Within 5 to 6 hours after landing on the flowering wheat spikes the macroconidia begin to germinate on the glumes. The airborne spores enter the flower through the base of palea and lemma, natural openings, or degenerating anthers to initiate infection (Bushnell et al., 2003). The germ tubes form a mycelial network on the glume surface (Pritsch et al., 2000). There are different routes for Fusarium hyphae to grow ad-axially on the palea and lemma during infection and pass through wheat florets. They can pierce the epidermal cell wall directly by a penetration peg, or pass through the stomata. The pathogenesis process begins as the hyphae penetrate the cuticle and start to grow underneath it. It takes about 76 hours for the hyphae to grow through the glume surface towards the developing caryopsis. The spread of the pathogen from one well colonized floret to another is a systemic and necrotic infection process, through the rachis and rachis nodes (Schroeder and Christensen, 1963; Ribichich et al., 2000). The vascular bundles within the rachis and rachilla facilitate the spread of the hyphae within the spike (Kang and Buchenauer, 2000; Ribichich et al., 2000). The tissue and organelles of host cells become fragmented due to the horizontal and vertical spread of the hyphae, which grows both basally and apically to spread throughout the rachis, until it reaches the peduncle of the spike (Kang and Buchenauer, 2000).

At the first stage of infection, fungal growth is asymptomatic and intercellular spread is through the pith and xylem (Bushnell et al., 2003; Guenther and Trail, 2005; Jansen et al., 2005). This stage is defined as biotrophic (Bushnell et al., 2003), although the lack of intracellular growth is in contradiction to traditional biotrophy (Jansen et al., 2005). Later, the fungus spreads radially and starts to grow intracellularly, colonizing the plant tissues, causing water soaking of chlorenchyma tissue. Subsequently, the water-soaked tissue becomes prematurely bleached, forming a band of several florets, often in the middle of the wheat spike (Trail, 2009).

2.2.2.3 Disease symptoms of Fusarium

Moist environmental conditions promote fungal colonization of immature wheat spikes and the formation of salmon orange to pink asexual spore masses (sporodochia)

at anthesis. The symptoms often appear first in florets which flower the earliest, usually located at the middle of the spike, but rapidly spread both basally and apically (Shaner, 2003; Bushnell et al., 2003; Osborne and Stein, 2007). Later on, the spikelets are filled with sexual ascospores produced within black perithecia (Osborne and Stein, 2007). The diseased kernels are chalky white, shriveled, light weight, and covered by pink mycelia and often referred as "tombstone" kernels or Fusarium damaged kernels (FDK) (Bushnell et al., 2003; Goswami and Kistler, 2004). The damage becomes visible as the crop matures, generally at the soft-dough stage of kernel development. FHB can reduce crop yield as much as 80% (McMullen et al., 2012).

2.2.3 Mycotoxin production and types

Mycotoxins are secondary metabolites, produced by fungal pathogens, which are toxic to plants, animals, and even human beings. They act as virulence factors that are necessary in the infection process or play a role as competitive weapons for purging other microbes during the process of infection, colonization, and spread in the host plants. The most important mycotoxins produced by *Fusarium* spp. are the trichothecenes, zearolenones, and fumonisins. Additionally, there are some minor mycotoxins, such as beauverins, fusarins, and moniliformins.

Trichothecenes are sesquiterpenoid compounds (Figure 2.2) characterized by a tricyclic 12, 13-epoxytrichothec-9-ene (EPT) core structure (Cole et al., 2003; Grove, 2007; McCormick et al., 2011). A cyclopentyl molecule is joined to the tetrahydropyran ring via C-2 and C-5; C-12 acts functionally as part of the epoxide, contributing to the toxicity of this compound (Cracraft, 1983). There are four types of trichothecenes: Type A, which include diacetoxyscirpenol, T-2 toxin, and HT-2 toxin; Type B, is mainly composed of nivalenol, deoxynivalenol, and the acetylated derivatives, 3-acetyl-deoxynivalenol (3ADON), and 15-acetyl-deoxynivalenol (15ADON). Type A and B are commonly associated with FHB. *Fusarium graminearum* and *F. culmorum* are generally Type A and B producers. *Fusarium sporotrichioides* and *F. verticilioides* are commonly moniliform producers. The Type C trichothecene is named Crotocin, and the Type D trichothecene is called Roridin E; however, Type C and D trichothecenes are not associated with FHB.

The greatest concern associated with FHB is the production of trichothecenes

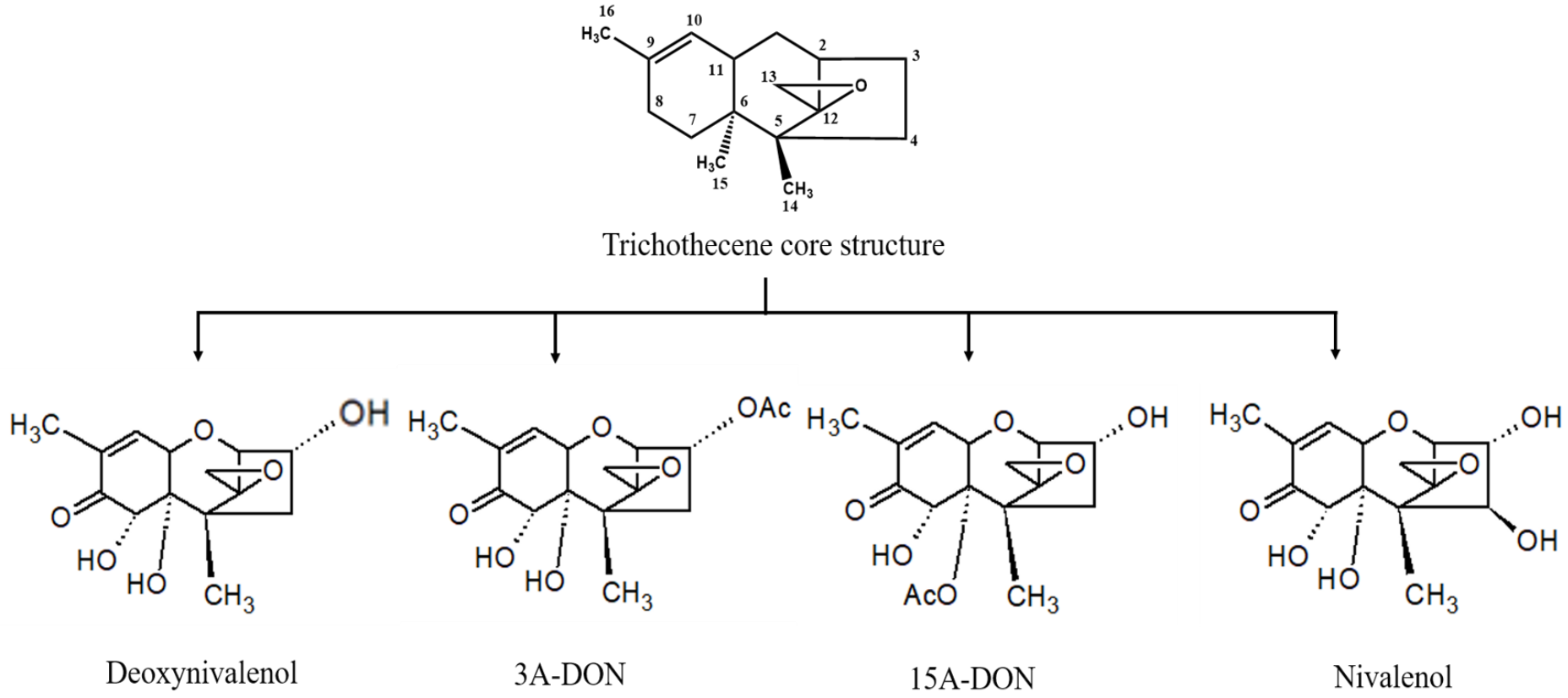


Figure 2.2: Structures of mycotoxins in this study. This figure includes the core structure of trichothecenes, and acetylated derivatives from this core trichothecene structure. In the second line, from left to right: Deoxynivalenol (DON), 3-Acetyl-Deoxynivalenol (3-ADON), 15-Acetyl-Deoxynivalenol (15-ADON), and Nivalenol (NIV).

by the pathogen. They are the key factors causing non-specific disease symptoms in plants, including chlorosis, necrosis, and wilting. Therefore, they are considered to be phytotoxic molecules (Desjardins, 2006; Nishiuchi et al., 2006), resulting in reduced crop yield and diminished grain quality. The synthesis of trichodiene is encoded by the *TRI5* gene of the fungus, which catalyzes the initial biosynthetic reaction to synthesize trichothecene. The expression and production of deoxynivalenol is directly associated with the expression of *TRI5 in vitro*. The aggressiveness of Fusarium isolates was associated with the function of the trichothecenes. Fungicide treatment of wheat plants increased the expression of *TRI5* gene (Desjardins, 2006).

Each Fusarium species produces its own unique spectrum of mycotoxin (Desjardins, 2006; Moretti et al., 2013). *Fusarium graminearum* isolates produce Type B trichothecenes, including DON and its derivatives 3-ADON, 15-ADON, and NIV, although no isolates of *F. graminearum* produce both DON and NIV. Fusarium culmorum and F. cerealis are also Type B trichothecene producers (Goswami and Kistler, 2005; Amarasinghe et al., 2015). Fusarium sporotrichioides and *F. poae* produce Type A trichothecenes, such as T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (Torp and Langseth, 1999). In cold temperate regions such as Canada and northern Europe, where *F. avenaceum* prevails, moniliformin and enniatins have been identified (Logrieco et al., 2002; Battilani and Logrieco; 2014; Ichinoe et al., 1983; Bacon et al., 1996; Bottalico and Perrone, 2002; Amarasinghe et al., 2015).

2.2.4 Methods to detect and quantify mycotoxins

Traditional methods for mycotoxin analysis (Gilbert and Pascale, 2014) include: high-performance liquid chromatography (HPLC) coupled with diodearray (DAD), ultraviolet (UV), fluorescence (FD), mass spectrometry (MS), or gas chromatography (GC) coupled with a flame ionization detector (FID), electron capture (ECD), or MS, and thin-layer chromatography (TLC) (Lippolis et al., 2008; Visconti et al., 2005) as well as enzyme-linked immunoassay (ELISA) (Wang et al., 2011; Zheng et al., 2005). Most of the methods have been optimized for accurate and precise mycotoxin identification in food samples, but these methods require skilled operators, extensive pretreatment of samples, and specific equipment. A major challenge is accuracy at very low toxin concentrations (Cigić and Prosen, 2009; Goryacheva et al., 2007); therefore, a routine analytical method is required that is sensitive, specific, rapid and can

determine very minute quantities of toxins in foods and beverages (Chauhan et al., 2016).

Both GC-MS and LC-MS are the most widely used methods to detect and quantify toxins. To-date LC-MS/MS methods have been widely developed, optimized and validated, an adopted in cereal matrices to determine concentrations of multiple mycotoxins simultaneously in various cereal-based food or feed products (Sanders et al., 2013; Jung et al., 2015; Oplatowska-Stachowiak et al., 2015; Zhang et al., 2016; de Luz et al., 2017; Miró-Abella, 2017; Kim et al., 2017).

Recently biosensors have been used to detect mycotoxins. The biosensors are easy to use, portable, rapid and above all easy to use. The detectors include biomolecules, which contain DNA, enzymes, and antibodies; as well as synthetic chemicals including molecular imprinted polymers (MIPs), mimotopes, and aptamers. When labeled with nanoparticles, enzymes, fluorophores, and redox molecules, mycotoxins that are small and neutral molecules can be easily detected. The biosensors can be classified into two groups: labeled and label-free groups. Within each group, sensors can be further categorized as competitive and non-competitive, based on the identification method (Chauhan et al., 2016). Surface plasmon resonance (SPR) spectroscopy is the most sensitive tool in small molecule identification without labelling. However, the SPR signal change is lower than the antibody signal, therefore the utilization of SPR spectroscopy in apta sensors for the analysis of mycotoxins has been rare (Wang et al., 2009; Chauhan et al., 2016).

2.2.5 Chemotypes and their importance in disease induction and spread

The toxicity of *Fusarium* spp. varies among the types of trichothecenes. Compared with Type B trichothecene such as DON and NIV, Type A trichothecenes such as DAS and T-2 are more toxic (Leeson et al., 1995; Amarasinghe, 2016). Although less toxic to plants, compared with DON, NIV has much higher toxic effects on livestock and humans (Schothorst and van Egmond, 2004; EFSA CONTAM Panel, 2013; Lee et al., 2015). Recently, *F. cerealis* was identified for the first time in Canada in FHB infected winter wheat spikes (Amarasinghe et al., 2013). All the *F. cerealis* isolates produce NIV that is less toxic than DON and 15-A DON in humans but has high toxicity in animals. The discovery of NIV producer *F. cerealis* will be of potential concern to Canadian wheat producers and the livestock feed industry (Amarasinghe et

al., 2013).

2.2.6 Chemotypes and disease forecasting

Several factors affect the FHB severity and DON accumulation. For instance, the differences between FHB severity and DON concentration may be due to the distinct pathogen population in different areas. FHB can be caused by many *Fusarium* spp., within which different pathogen isolates may produce different spectrum or amounts of toxins reflecting the Fusarium population (Gale, 2003; de Wolf and Paul, 2014). Some of these variable factors may be related to geography or correlated with climatic patterns or cropping systems (de Wolf and Paul, 2014). Therefore, developing a warning system for increased risk of FHB infestation and contamination of mycotoxin in wheat can give producers a response time to develop strategies to reduce the FHB losses. It will also give the grain producers and users preparation time to ameliorate potential exposure to humans or livestock in the agricultural industry (de Wolf and Paul, 2014).

The FHB prediction model, known as "DONcast" developed at the University of Guelph (Hooker et al., 2002), and has been utilized in several regions in North America, Europe, and South America. DONcast is a powerful tool that incorporates estimates of DON contamination for site-specific conditions enhancing prediction ability and accuracy with regard to susceptibility of cultivars, tillage, and crop rotations (Pitblado et al., 2007; de Wolf and Paul, 2014). In the USA, 30 states use the prediction system to estimate FHB outbreaks and DON accumulation in harvested grain (de Wolf and Isard, 2007; de Wolf et al., 2004; McMullen et al., 2012; de Wolf and Paul, 2014).

Like most of the plant diseases, wheat FHB is a complex disease, and includes several aspects of the host, pathogen, environment, and the interactions between them. Therefore, it is imperative to build a comprehensive knowledge base regarding different aspects of wheat FHB to develop strategies to control this devastating disease and achieve food and nutritional security (Chakraborty and Newton, 2011; Beres et al., 2018).

2.3 Strategies to manage FHB in wheat

The most effective strategy to mitigate the devastating effects of FHB includes a holistic approach combining genetic, environment and management tools (Bai and Shaner, 2004; Beres et al., 2018). The combination of breeding and cultivation of

resistant cultivars, cultural practices such as tillage, crop rotation methods, fungicide use, and the development of biological control agents have been able to reduce FHB incidence and overcome some of the damage due to FHB (Parry et al., 1995; Edwards et al., 2001; Mesterházy, 2003; Gilbert and Tekauz, 2011). Unfortunately, there are very few cultivars that can be classified as resistant to FHB. Therefore, there is a need to develop FHB resistant wheat cultivars (Beres et al., 2018).

2.3.1 Chemical control

Timely application of fungicides is part of an integrated FHB management system. The triazole fungicides, such as prothioconazole, tebuconazole, and metconazole are the most effective in reducing FHB effects (Edwards et al., 2001; Simpson et al., 2001; Pirgozliev et al., 2002; Mesterházy, 2003) and do so by inhibiting demethylation. They are effective against a broad spectrum of fungal diseases, such as leaf spots, rusts, and powdery mildew (Haeuser-Hahn et al., 2004; Mesterházy, 2014). The selection of fungicides and the application dose should take various factors into consideration: the resistance of the cultivar, weather conditions, the risk of severe disease in the field, crop sensitivity to fungicides and yield potential (Mesterházy, 2003). Multiple fungicide applications have added protective effect against FHB development and DON accumulation (Edwards and Godley, 2010; Yoshida et al., 2012).

Application timing and coverage of spikes by the fungicide are two important factors to be considered for fungicide application. To prevent infection of susceptible wheat cultivars, especially for those seeded after maize, a series of fungicide applications have been suggested, for example, the first application begins at anthesis, or traditionally recommended when 75% spikes of the main stems have emerged, followed by three applications up to 30 days after anthesis (Yoshida et al., 2012; Beres et al., 2018).

2.3.2 Cultural practices

Agronomic practices have been used to minimize yield loss due to FHB infestation. Several management strategies have been used to mitigate the disease and DON contamination in the grain. Some of the diesease management strategies include the use of wheat varieties with certain level of resistance to infection, planting seeds without any pathogen contamination, crop rotation, choosing type of tillage,

management of crop residues, timing of harvest, and avoiding mechanical damage or contamination during harvest and processing. In particular, efficiency of managenment can be improved if given high importance of consideration of the cropping history, plowing to bury the wheat residues, at least four-year rotation (Kutcher et al., 2011, 2013) of another crop, especially oilseed or pulse crops such as yellow mustard, field pea, alfafa, or canola, which is not a host of FHB, between wheat (Bai and Shaner et al., 1994; Milus and Parsons, 1994; Parry et al., 1995; Paulitz, 1996; Fernando et al., 1997; McMullen et al., 1997; Arseniuk et al., 1999; Beres et al., 2018).

2.3.3 Types of FHB resistance

Wheat genotypes vary in their response to FHB infection from 'susceptible' (S) > 'moderately susceptible' (MS) > 'intermediate' (I) > 'moderately resistant' (MR) > 'resistant' (R). Most current wheat cultivars are in the S and MR categories (Beres et al., 2018). There are five types of physiological resistance to FHB (Mesterházy, 1995): (I) resistance to initial infection, (II) resistance to spread within the spike, (III) resistance to infection of the kernel, (IV) tolerance during which infection is present but no substantial effect on grain yield, and (V) decomposition or non-accumulation of mycotoxin. Type I refers to resistance to initial infection, which is evaluated as the incidence of disease under natural field environment or artificial inoculation conditions (e.g., spray inoculations). Type II is the spread of disease within the spike after single floret inoculations (Figure 2.3). Wheat cultivars incorporating type I and type II resistance are preferred as the FHB resistance will be more stable and durable.

2.3.4 Genetics of resistance

The resistance of wheat to FHB is governed by multiple genes making it a complex quantitative trait (Buerstmayr et al., 2009). Several QTL located on many wheat chromosomes have been have been associated with FHB resistance (Buerstmayr et al., 2009; Cativelli et al., 2013). In a meta-analysis of 249 FHB resistance QTL present in 46 unique lines from 45 studies identified 209 QTL conditioning the four types of FHB resistance (Liu et al., 2009). The 209 QTL were classified into 43 clusters on 21 chromosomes. Of 209 QTL, 119 were significant and 116 explained more than 10% phenotypic (FHB resistance) variation (Liu et al., 2009). A recent review lists 250 QTL for FHB resistance, but most of these have small effects and some are still to be

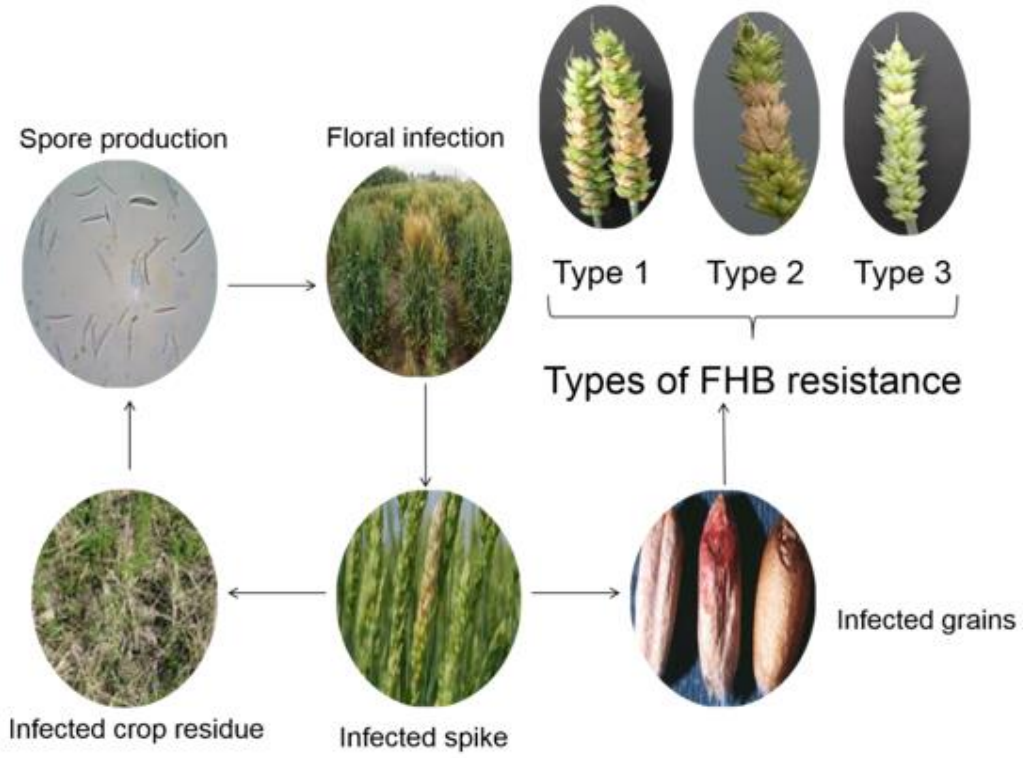


Figure 2.3: FHB infection pathway and three types of FHB resistance. Type I: resistance to initial infection; Type II: resistance to spread within the spike; Type III: resistance to deoxynivalenol (DON) accumulation.

verified (Jia et al., 2018). The four major QTL, *Fhb1* (Waldron et al.,1999; Buerstmayr et al., 2002), *Fhb2* (Cuthbert et al., 2007); *Fhb4* (Xue et al., 2010) and *Fhb5* (Xue et al., 2011) have been fine mapped. The *Fhb1* (syn. *Qfhs.ndsu-3BS*) is a major FHB resistance QTL for Type II resistance, which has been mapped on the distal fragment of 3BS of spring wheat. The resistant wheat cultivar Sumai-3 and Nyubai have been credited as sources for this QTL (Cuthbert et al., 2006). The QTL *Qfhs.ndsu-3BS* was correlated with mycotoxin accumulation (Lemmens et al., 2005). It was shown that this QTL encodes or regulates the expression of deoxynivalenol (DON) glucosyltransferase, which catalyzes the transfer of a glucoside moiety onto C-3 of DON to synthesize non-toxic D3G. Therefore, plant breeding for resistance to FHB in wheat has focused on the incorporation *Qfhs.ndsu-3BS* to introduce FHB resistance.

2.3.5 Candidate genes conferring resistance

Much effort has been made to identify and characterize QTL conferring FHB resistance. Transcriptome analysis using RNA-seq technology of three FHB resistant lines (Nyubai, Wuhan 1 and HC374) and a susceptible cultivar (Shaw) of wheat identified 37,772 differentially expressed genes, including 28,961 from wheat and 8811 from the pathofen (Pan et al., 2018). The three resistant genotypes, 220 differentially expressed genes were associated with FHB resistance. The two genotypes, HC374 and Nyubai, have a common FHB resistant QTL on 3BA and 5AS and these had similar transcriptomes compared to Wuhan 1, which carries the FHB resistant QTL on 2DL (Pan et al., 2018). The difference in expression profiles among resistant genotypes suggests a genotype-specific defence mechanism (Pan et al., 2018). Map-based positional cloning of *Qfhs.ndsu-3BS,* also known as *Fhb1,* from Sumai-3 identified a pore-forming, toxin-like (PFT) gene that conferred FHB resistance (Rawat et al., 2016). PFT encodes a chimeric lectin with two agglutinin domains and an ETX/MTX2 toxin domain, but the biochemical mechanism conferring FHB resistance is still unknown (Rawat et al., 2016). Mutation, gene silencing and transgenic over-expression studies provide evidence in support of the role of PFT in conferring FHB resistance. Independent of PFT, but in close proximity to it near the same genetic block, is a *UDP-glucosyltransferase* (UGT, EC 2.4.1.x) gene that catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at carbon-3 of deoxynivalenol leading to production of non-toxic DON-3-O-glucoside (D3G) (Wetterhorn et al., 2016). The UGT

mediated DON glycosylation is related to Type II resistance in Sumai-3 (Lemmens et al., 2005). Induced expression of *TaUGT2* and repressed expression of *TaUGT1* are associated with FHB resistance (Lin et al., 2008). Therefore, the *TaUGT2* gene may participate in resistance to FHB as well as resistance to DON. The *TaUGT3* gene, another member of the UGT gene family, has been cloned and analyzed from the FHB resistant landrace Wangshuibai. The overexpression of this gene leads to enhanced resistance to DON in Arabidopsis. The *TaUGT-2* and *TaUGT-3* genes have been mapped to the short arm of chromosome 3 (Lulin et al., 2010). Heterologous overexpression of barley UGT gene *Hv13248* in susceptible wheat cultivars induced FHB resistance both under field and greenhouse conditions (Li et al., 2015).

Ethylene signalling plays a key role in disease resistance of dicotyledonous plants. In Arabidopsis, mutants with a lower level of ethylene signalling were more resistant to *F. graminearum*, while the mutants with enhanced ethylene signalling were more susceptible; similar results were found in wheat and barley (Chen et al., 2009). Therefore in wheat, decreased expression of the ethylene insensitive 2 (*EIN2*) gene reduced disease symptoms and DON concentration (Chen et al., 2009). In both monocotyledonous and dicotyledonous plants, *F. graminearum* affects ethylene signalling (Chen et al., 2009) suggesting that identification of wheat germplasm with variation in ethylene signalling could be another strategy to improve FHB resistance.

Two genes, *TaABCC3.1* and *TaABCC3.2*, variants of an ATP-binding cassette (ABC) transporter gene, were isolated from DON-treated wheat mRNA. Among the three homologs, *TaABCC3.1* was identified as the variant related to DON resistance in wheat (Walter et al., 2015). This has been suggested as as another candidate gene for FHB resistance.

Recently, a metabolomics approach has been used to identify and characterize resistance related (RR) metabolites with the eventual objective of identifying candidate genes. A similar approach has been used to identify RR metabolites in barley (Bollina et al., 2010; Kumaraswamy et al., 2011) and wheat (Gunnaiah et al., 2012). An integrated metabolomics strategy combined with gene sequence information, gene expression, histochemical studies and heterologous gene expression identified agmatinecoumaroyl transferase (ACT) as a candidate gene located at FHB QTL-2DL (Kage et al., 2017). The wheat TaACT was present in the NIL-R and its role in FHB resistance was confirmed by virus induced gene silencing in Arabidopsis (Kage et al.,

2017). In summary a number of candidate genes involved in FHB resistance have been identified, although the precise role of these genes in FHB resistance needs to be defined.

2.3.6 Sources of FHB resistance

Several genomic regions on the wheat chromosomes are associated with FHB resistance thus implying it is controlled by many genes, each with a small effect. This also suggests that a range of resistance sources can be used as sources of FHB resistance. The Chinese wheat cultivar 'Sumai 3' has been used to develop FHB resistant wheat lines (Buerstmayr et al., 2003); it was developed by the Taihu Regional Institute of Agricultural Sciences located in the Lower Yangtze River region where FHB occurs frequently and severely because of the warm and humid conditions during flowering (Jia et al., 2018). The best FHB resistant QTL that have been validated are: *Fhb1*, *Fhb2* and *Qfhs.ifa-5A*, all derived from Sumai 3 (Waldron et al., 1999; Bai et al., 1999; Anderson et al., 2001; Buerstmayr et al., 2002, 2003a,b). Another Chinese cultivar, Wangshiubai, has FHB resistance similar to Sumai 3 and has been a source of *Fhb4* and *Fhb5* (Xue et al., 2010; 2011). Other characterized sources of FHB resistance include *Qfhs.nau-2DL,* identified from breeding line CJ9306 (Jiang et al., 2007a,b) and *Fhb7* from the wild species *Thinopyrum ponticum* (Guo et al., 2015). The Brazilian cultivars Frontana, Encruzilhada, and Arotana confer Type I resistance and have been used as sources of FHB resistance (Mesterházy, 1997).

2.4 Evaluation of FHB

2.4.1 Field methods

Screening for FHB resistance is a critical to identifying FHB resistant germplasm and the selection of FHB resistant lines from the progeny of crosses in a typical breeding program. The evaluation of FHB is a critical factor that needs to be performed accurately and precisely. At present, field-based inoculation (to the soil surface and into single florets) and other approaches including the detached leaf assay, the clip dipping method, the foliar spray method and point inoculation have been reportedly used to evaluate FHB severity or resistance (Kumar et al., 2011; Shin et al., 2014; Yang et al., 1999). Field based FHB screening is reliable, but is affected by environmental conditions, and is time and labor intensive.

2.4.2 Laboratory methods

Several laboratory-based FHB screening methods including clip dipping, foliar spray, and point inoculation have been optimized and reported (Shin et al., 2014). With the clip dipping method, stems of three-day old seedlings are wounded by pin prick and inoculated with a fungal suspension, whereas clip dipping and foliar spray methods screen 10-day old seedlings with fungal suspension using dipping and spray methods, respectively. These are indirect methods for FHB screening and have not been validated with field experiments, therefore their use for germplasm screening may influence the selection process in breeding programs.

2.5 High resolution melting curve identification of single nucleotide polymorphisms (SNP)

Single nucleotide polymorphism (SNP) is commonly used as markers for genotyping. Conventional SNP typing methods require several probe-based assays that need to be multiplexed or need the use of DNA microarrays. There are a wide range of approaches that can be applied in SNP genotyping. These technologies, which include the gel-based methods are relatively low-throughput, such as cleaved amplified polymorphic sequence (CAPS) (Thiel et al., 2004). High-throughput PCR-based approaches involve labeling with fluorescence such as the high resolution melt (HRM) curve assays, in addition to KASP^{TM} array and TaqMan^{R} (Martino et al., 2010). Array based technologies are useful when whole genome SNP typing is needed. HRM is a very fast and cost-effective genotyping method for SNP detection based on the analysis of melting curves of PCR products. The HRM analysis (Ririe et al., 1997) is the natural extension of real-time PCR monitoring. It uses intercalating fluorescent dyes to monitor the dissociation behavior of double-stranded DNA in response to increasing temperature. The GC content of DNA and the overall distribution of bases affects the dissociation (melting) of DNA. The AT-rich regions melt faster than GC-rich regions. Due to its high efficiency in mutant detection, HRM has been widely used in many studies to identify SNPs, and deletions/insertions (IN/DELs) in targeting induced local lesions in genome (TILLING) populations with complicated crop genomes (Figure 2.4). HRM has been successfully used to genotype mutants with single mutations (Lochlainn et al., 2011). In a recent study, HRM was used to show the association of the B genome

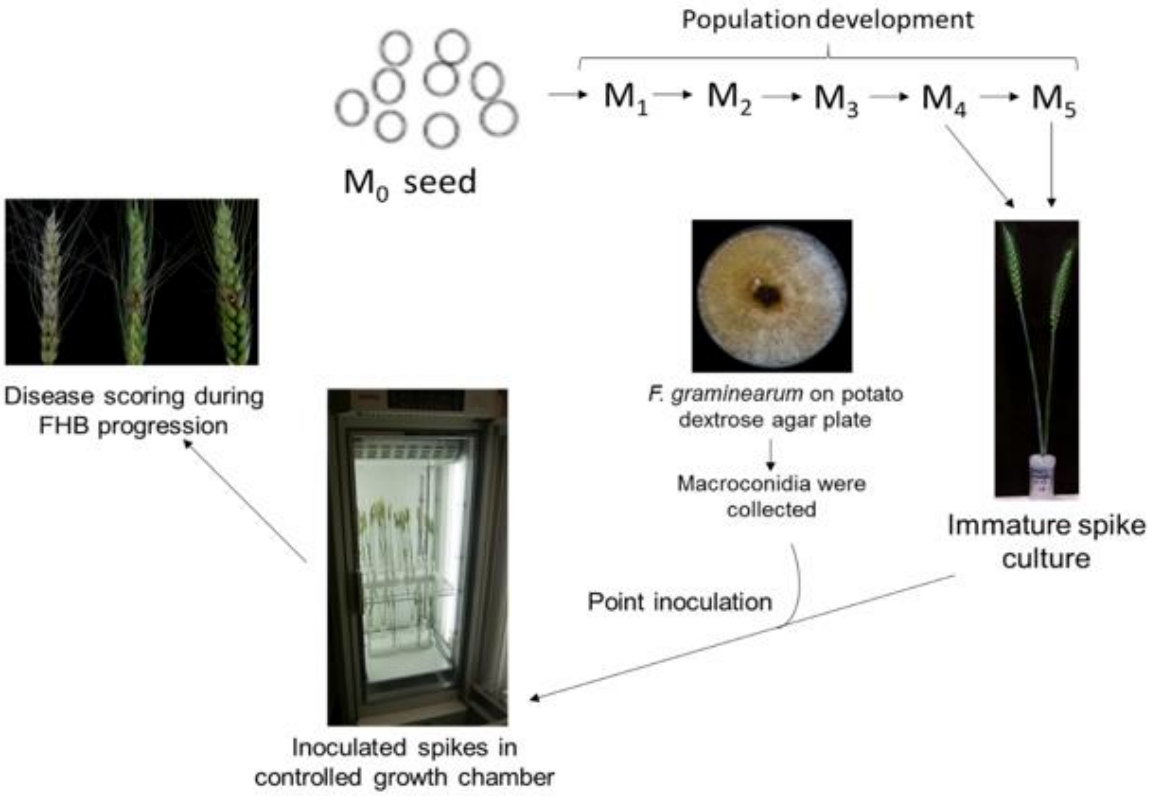


Figure 2.4: Work flow for the creation, screening and phenotyping of wheat plants derived from SCDV wheat TILLING population of plants based on the immature spike culture method.

specific *TaUGT-2* and *Ta-UGT-3* genes in FHB resistance in wheat (Sharma et al., 2018).

2.6 Concluding remarks

To summarize, FHB is a complex cereal crops disease that reduces grain yield resulting in major revenue loss to the producers. FHB infection produces mycotoxins in grains and adversely affects human and livestock health. To mitigate the deleterious effects of FHB, a comprehensive strategy has been suggested that incorporates genetics, management and production in low risk environments (Beres et al., 2018). The non-availability of wheat cultivars with durable resistance to FHB is a major challenge to wheat geneticists and plant breeders. An efficient FHB screening technique to identify and characterize FHB resistant germplasm will help to expedite the development of wheat cultivars with durable FHB resistantce. A recently developed immature spike culture to screen FHB resistance, identification of new FHB resistant wheat germplasm and characterization of a SNP in *UDP-glucoyltransferase* associated with FHB resistance described in the following chapters will help in the development of wheat cultivars with durable FHB resistance.

CHAPTER 3. OPTIMIZATION OF SPIKE CULTURE METHOD TO SCREEN FUSARIUM HEAD BLIGHT RESISTANT WHEAT AND ASSOCIATED SINGLE NUCLEOTIDE POLYMORPHISMS IN B-GENOME SPECIFIC *UDP-GLUCOSYL TRANSFERASE*

Study 1

This study was conducted to optimize the immature spike culture technique to assess FHB. The second objective of this study was to develop molecular markers associated with FHB resistance. The results from this chapter were included in two publications Sharma et al 2018 and Huang et al (submitted).

CH contributed to the FHB screening described in Sharma et al. (2018).

CH conducted the experimental work described in Huang et al.

Sharma, P., Gangola, M. P., **Huang, C.**, Kutcher, H. R., Ganeshan, S. and Chibbar, R. N. (2018). Single Nucleotide Polymorphisms in B-Genome Specific *UDP-Glucosyl Transferases* Associated with *Fusarium* Head Blight Resistance and Reduced Deoxynivalenol Accumulation in Wheat Grain. *Phytopathology* 108(1), pp.124-132.

Huang, C., Gangola, M.P. and Chibbar. R.N. Wheat in vitro spike culture screening of Fusarium Head Blight infection revealed a positive correlation between disease severity and mycotoxin accumulation using LC-MS/MS. Can J Plant Pathol (submitted).

3.1 Abstract

In vivo methods commonly used to screen fusarium head blight (FHB) disease development in wheat is complex, time consuming and subject to environmental effects therefore a rapid laboratory-based method using a spike culture method was developed to screen EMS mutants of wheat for FHB resistance. Using this method, statistically signidicant differences were detected in FHB disease development between AC Nanda, Sumai-3 and 134 EMS mutant lines. AC Nanda had 54% disease severity whereas Sumai-3 had 35% disease severity. Among 134 EMS mutant lines, 65 had disease severity between 0% and 35% whereas 47 lines had disease severity range from 54% to 100%.

To understand the molecular mechanism underlying FHB resistance, genomic DNA corresponding to *TaUGT-2* (B genome), *TaUGT-3* (B genome) and one of the *TaUGT-EST* (D genome) which are known to participate in FHB resistance were cloned and sequenced from AC Nanda and Sumai-3. Nucleotide sequence comparison revealed one nucleotide change in each of these genes between AC Nanda and Sumai-3. These changes were located in the exonic regions of these genes and were synonymous for *TaUGT-2* and *TaUGT-3* but created a premature stop codon in *TaUGT-EST*. EMS mutant lines were screened for mutations in these genes using high resolution melt (HRM) analysis and validated by sequencing. Statistical analysis determined that the nucleotide difference present at position 450 and 1558 bp from translation initiation site between AC Nanda and Sumai-3 in *TaUGT-2B* and *TaUGT-3B* gene, respectively, correlate significantly ($P<0.01$) with the disease severity of EMS mutant lines. This study not only established spike culture as a tool to screen FHB resistance and associated mutations in *TaUGT-2B* and *TaUGT-3B* with FHB resistance in wheat but also identified FHB-resistant lines which can be used in breeding programs as a source of FHB resistance.

3.2 Introduction

Fusarium head blight (FHB) or scab, is a potentially destructive fungal disease of cereal grain crops, with worldwide economic and health impacts (Bai and Shaner, 1994; Parry et al., 1995; McMullen et al., 1997). The disease is caused by a range of trichothecene-producing Fusarium species among which *Fusarium graminearum* (teleomorph: *Gibberella zeae*) is the most economically relevant (Parry et al., 1995).

Wheat and barley are the two most heavily FHB-affected crops and suffer the largest economic damage. FHB not only affects the yield and quality of grains but also causes contamination of infected grains with trichothecene mycotoxin, especially deoxynivalenol (DON) and nivalenol (NIV) which prevent its use for human consumption or feed (Bai and Shaner, 1994; Ichinoe et al., 1983; Jansen et al., 2005; Foroud and Eudes, 2009; Cho et al., 2012). Trichothecenes such as DON, produced by *F. graminearum* are acutely phytotoxic and act as virulence factors on sensitive host plants. In livestock, deoxynivalenol is linked to feed refusal (Meronuck and Xie, 2000) whereas in humans it may cause gastrointestinal symptoms including vomiting and depression of the immune system (Maresca et al., 2002; Pestka et al., 2004; Bryden, 2007). Direct and secondary economic losses of $2.7 billion were estimated for all crops in the northern Great Plains and central US from 1998 to 2000 (Nganje et al., 2002).

In wheat, a major quantitative trait locus *Qfhs.ndsu-3BS* for FHB resistance was identified on the short arm of chromosome 3B (Waldron et al., 1999). *Qfhs.ndsu-3B* was later designated as *Fhb1* (Liu et al., 2006). Wheat lines segregating for *Fhb1* (a major quantitative trait locus for FHB resistance) exhibited an increased DON-glucoside/DON ratio after treatment with DON, implying that the metabolism of DON had a significant role in the FHB resistance complex (Lemmens et al., 2005). The authors proposed that *Fhb1* may encode a member of the *UDP-glucosyltransferase* gene family, which can catalyze the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of deoxynivalenol leading to production of non-toxic DON-3-Oglucoside (D3G) or a regulator of a *UDP-glucosyltransferase*. Multiple candidate *UDP-glycosyltransferase* (*UGT*) genes from different crop plants that are either induced by Fusarium spp., DON treatment or differentially expressed in cultivars varying in Fusarium disease resistance have been described. In young spikes of bread wheat infected by *F. graminearum*, Lin et al. (2008) observed induced expression of *TaUGT2* but *TaUGT1* expression was repressed suggesting that *TaUGT2* may participate in wheat resistance to FHB in which mycotoxin DON plays a role. A DON-resistance related gene (*TaUGT3*) was also cloned and characterized from a FHB resistant wheat (variety Wangshuibai, overexpression of which led to enhanced tolerance against DON in Arabidopsis (Lulin et al., 2010). Keeping in mind the importance of *TaUGT-2*, 3 and *TaUGT-EST*, in FHB resistance, mutations in these genes were studied in AC Nanda, Sumai-3 and EMS mutant lines and efforts were made to

correlate mutations in these genes with FHB resistance.

To assess the severity of Fusarium head blight disease, point inoculation method is commonly used. Since not all varieties and plants within the same variety flower simultaneously, some experimental variation between early and later flowering plants may exist. Moreover, final observation for this disease is generally taken after 21 days of inoculation which is time consuming. Therefore, a rapid, uniform, reliable and laboratory-based method needs to be developed for FHB screening.

In the present study, *in vitro* spike culture technique (Ganeshan et al., 2010) was optimized to screen wheat genotypes for FHB resistance. The analyses of nucleotide sequences of *TaUGT* discovered gene sequence variants and identified SNP associated with FHB resistance in wheat.

3.3 Materials and Methods

3.3.1 Development of spike culture as a tool to screen for FHB resistance

Experiments were conducted to establish spike culture as a tool to screen for fusarium head blight resistance in wheat. In order to choose the optimum concentration and time to administer *F. graminearum* isolates for screening, 15 µL of 10^3, 10^4, 10^5 and 10^6 macroconidia/mL of four *F. graminearum* isolates FGSC-9075, M1-07-2, M7-07-1 and M9-07-1 (kindly provided by Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg) were used to point inoculate each of two basal florets of a spikelet positioned in the middle of the spike of wheat cv. AC Nanda after 0, 2, 5, 8, 11 and 14 days in culture. Isolate FGSC 9075 is a wild type fusarium which has full genomic information available. FGSC-9075 and M1-07-2 are 15-acetyl-deoxynivalenol (15-ADON) chemotypes whereas M9-07-1 and M7-07-1 are 3-ADON chemotypes.

The soft white spring wheat cv. AC Nanda plants were grown in a growth chamber in pots containing Sunshine Professional growing mix (Sun Gro Horticulture, Canada Ltd.) at 20˚C/16 h light (350 µmol m^{-2} s^{-2} PPFD) and 18˚C/8 h dark. Plants were fertilized every 3 weeks with slow release fertilizer, Nutricote-14-14-14: N-P-K (Plant Products Co. Ltd., Brampton, ON, Canada). Main tillers bearing immature spikes were cut above the soil surface on the day spikes emerged completely (Feekes scale, 10.5; Haun scale, 11). All the leaves and flag leaves from spikes were removed and placed in tubes containing 5 mL of culture medium consisting of 50 g L^{-1} sucrose and 0.4 g L^{-1} L-glutamine, buffered with 0.5 g L^{-1} morpholino ethane sulfonic acid (Ganeshan et al.,

2007). Medium pH was adjusted to 6.2. The spikes were transferred to fresh culture medium every 3 days. Macroconidial suspensions of isolates were prepared by washing 7-day-old potato dextrose agar cultures with 5 mL of sterile distilled water. Macroconidia were then dislodged using a sterilized glass rod. Suspensions were filtered through 4 layers of cheese cloth to remove large sections of mycelium and other debris. Macroconidia concentrations were determined microscopically using haemocytometer and were adjusted to10^6 macroconidia/mL using sterile distilled water. Serial dilutions were then made to achieve concentrations of 10^5, 10^4 and 10^3 macroconidia/mL. Final concentrations were also verified microscopically. To determine inoculum viability, 15 µL of inoculum was plated on water-agar media. In all cases, >99% of macroconidia germinated. Tween-20 (0.02% v/v), a nonionic surfactant, was added as a wetting agent in the preparation of conidial suspensions. The macroconidial suspension was injected between the lemma and palea of a basal floret positioned in the middle of spike. Control spikes were inoculated with sterile distilled water amended with 0.02% Tween-20 (v/v). The inoculated spikes were covered with clear plastic bags misted with distilled water for 3 days to increase humidity and promote infection and were kept in a Sanyo Versatile Environmental Test Chamber (Sanyo MIR-351H, Canada) at 16-h day and 8-h night cycle, temperatures 24°C (night) and 26°C (day). After 3 days, bags were removed, and disease was allowed to develop. Spikes were rated every alternate day after 5 DAI for disease development until bleaching started due to natural senescence (aging). The numbers of bleached and total spikelets were counted for each treated spike in culture to calculate disease severity (number of diseased spikelets/total) of disease. All experiments were performed in triplicate.

3.3.2 Mutant population generation, screening for FHB resistance and SNP

A spike culture system was used to generate an EMS mutagenized population of wheat (Ganeshan and Chibbar, 2017). Briefly, immature spikes were treated with EMS concentration of 0.25% (w/v) for 4 h to generate the initial M_0 population. Seeds were germinated to produce M_1 plants for which each spike was maintained separately. M_1 plants were self-fertilized to produce M_2 seeds. Seeds from each M_2 spike were also kept separately. As EMS treatment was applied in culture medium prior to anthesis, the M_2 population was expected to be less heterozygous. Three seeds from three spikes of

each M_2 plant were planted to produce M_3 plants. Leaf samples from M_3 plants around 20 day were collected for DNA extraction. The immature spikes were used to screen for FHB resistance using 15 µL of 10^5 macroconidia/mL of M7-01-1.

3.3.3 PCR amplification of *UDP-glucosyltransferase* (*UGT*)

Genomic DNA extraction from leaves was performed using a Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The oligonucleotide primers were designed for the amplification of *UDP-glucosyltransferase* (*UGTs*) *TaUGT-2* (GenBank Accession EU568801.1) and *TaUGT-3* (GenBank Accession FJ236328.1). The primers were then tested for genomic specificity in a PCR reaction by using DNA of wheat genomes A (*T. monococcum*), B (*A. speltoides*), D (*A. tauschii*) respectively. Common wheat cultivars AC Nanda and Sumai-3 were also used as susceptible and resistant controls. The sequence similarity of the nucleotide sequences were assessed against the non-redundant genes/transcripts in NCBI database (http.//www.ncbi.nlm.nih.gov/BLAST) by deploying the BLASTN algorithms. The alignment of multiple sequences was performed to discover nucleotide variations or SNPs between the resistant and susceptible wheat lines by using CLC genomics workbench 4 software (CLC Bio, Cambridge, Massachusetts, USA) (Sharma et al., 2018).

3.3.4 Mutation detection in *UDP-glucosyltransferase* genes using DNA Sequencing and HRM

For mutation screening a high resolution melt (HRM) assay was performed using genomic DNA extracted from leaves of M_3 plants. Nested PCR approach was used to amplify sequence specifically from the B genome of *TaUGT-2* and *TaUGT-3*. HRM reaction was first performed for *TaUGT-2* and *TaUGT-3* using B genome specific primers *TaUGT-2F1/2R2* and *TaUGT-3F1/3R2* (Table 3.1) respectively to amplify genomic DNA, prior to using the HRM primers with this product. Oligo deoxynucleotide primers (forward and reverse) for target genes *TaUGT-2B* and *TaUGT-3B* were designed to target the mutation located at 450 bp and 1558 bp, in which AC Nanda and Sumai-3 differ (Table 3.1). D genome specific primer (*TaUGTHF3/HR3*) was used to perform the HRM assay for *TaUGT-EST* directly from genomic DNA (Table 3.1). Four more primer pairs *TaUGT-2BHF2/HR2*, *TaUGT- 2BHFF/HRE*,

Table 3.1: Details of primers used for gene isolation and HRM analysis to cover the region where AC Nanda and Sumai-3 differ.

Primer	Gene/EST	Purpose	Accession # /Source	Sequence 5' to 3'
TaUGT-2BF1	*TaUGT-2B*	Gene isolation	EU568801.1	CACTTTTGCTGCCACAGC
TaUGT-2BR2	*TaUGT-2B*	Gene isolation	EU568801.1	AGAAAAGGCGAGCTTATTACCG
TaUGT-3BF1	*TaUGT-3B*	Gene isolation	FJ236328.1	TCCACTAGCACTTCAGCCG
TaUGT-3BR2	*TaUGT-3B*	Gene isolation	FJ236328.1	CTGAAAAAGCGAGCTTAGGTAA
TaUGT-EST-F1	*TaUGT-EST*	Gene isolation	FG985273.1	AGCTTGAGGTTCTTGCACATA
TaUGT-EST-R1	*TaUGT-EST*	Gene isolation	FG985273.1	CAGCGATATTCTGGTCTGAACT
TaUGT-2B Fd	*TaUGT-2B*	HRM analysis	AC Nanda	CGCCTCCTAGCTGCATCATATC
TaUGT-2B Rd1	*TaUGT-2B*	HRM analysis	AC Nanda	GCCCAGTGCTCACAAGGTT
TaUGT-3B HF1	*TaUGT-3B*	HRM analysis	AC Nanda	CCGTTGGAGGATTCGTGACGC
TaUGT-3B HR1	*TaUGT-3B*	HRM analysis	AC Nanda	CATTCTCAACTCCTCTGTAGCCT
TaUGT EST HF3	*TaUGT-EST*	HRM analysis	AC Nanda	GGTTGCTTTGTTACCCAT
TaUGT EST HR3	*TaUGT-EST*	HRM analysis	AC Nanda	GTCTGAACTTCCTCCTTCCT

TaUGT-2BHFO/HRZ and *TaUGT-2BFH/RH* (Table 3.1) were designed to target 4 mutations at 267, 653, 734 and 1041bp respectively in the *TaUGT-2B* gene in the EMS mutant population (Table 3.1).

Amplification was performed in a total reaction mixture of 20 µL containing 10 µL of MeltDoctor HRM Master Mix (Applied Biosystems, Inc., CA, USA), 0.3 µM of each primer, 1 µL PCR product as DNA template and sterile deionized water using a 7500 Fast real-time PCR system (Applied Biosystems, Inc., CA, USA). Control samples without DNA were included in each PCR run. The PCR cycling started with an initial phase of 10 min at 95°C, then 40 cycles of 15 s at 95°C (denaturation step) and 60°C for 30 sec (annealing and elongation steps). HRM was carried out immediately following PCR from 60°C to 90°C at steps of 0.1°C, each step with a 10 s hold. The program consisted of denaturation at 95°C for 10 sec, 60°C for 1 min (annealing), 95°C for 15 sec (high resolution melting) and final annealing at 60°C for 1 min. The high resolution melting curve profile was then analyzed using HRM analysis software version 2.0.1. EMS mutants with nucleotide variation were easily distinguished by plotting the fluorescence difference between melting curves. HRM results were validated by DNA sequencing of 60 independent PCR products including AC Nanda and Sumai-3 and 58 EMS mutant lines.

The PCR system for the amplification for *TaUGT* is described as below. In total 25 µL PCR reaction volume, it contains 2.5 µL 10×Dream Taq buffer, 2 µL 25 mM $MgCl_2$, 0.5 µL 10 mM dNTP mix, 0.125 µL Taq polymerase, 1 µL DMSO, and 15.875 µL ddH_2O. Besides, 1 µL 5 mM forward primer, 1 µL 5 mM reverse primer, as well as 1 µL diluted DNA template of the target sample were also included in this reaction system. The PCR cycles were programmed on a Thermocycler (Eppendorf, Germany) and conducted as follows. The initial denaturation phase of 2 min at 95 ℃, followed by 35 cycles consisting of a denaturation phase of 30 seconds at 94 ℃; holding at 60 ℃ for primer annealing for 30 seconds, followed by an elongation step for 1 min at 72 ℃. At the end, a final extension time of 10 min at 72 ℃ was applied.

3.3.5 Statistical Analysis

Analysis of variance (ANOVA) was used for the identification of the statistically significant data. The ANOVA results were checked using Tukey's test using Minitab software (Version 16) (Minitab, Inc., Pennsylvania, USA). Association tests

were used for detecting the association between the nucleotide variants and the FHB disease phenotypes using SAS 9.4® (SAS Institute Inc. Cary, NC, USA).

3.4 Results

3.4.1 Optimization of *in vitro* immature spike culture method to identify FHB severity differences between resistant and susceptible wheat genotypes

To establish spike culture as a screening tool for FHB resistance, spikes of wheat cv. AC Nanda were point inoculated with 15 µL of 10^6, 10^5, 10^4 and 10^3 macroconidia/mL of four *F. graminearum* isolates FGSC-9075, M1-07-2, M7-07-1 and M9-07-1 after 0, 2, 5, 8, 11 and 14 days in culture, natural senescence (ripening) started in control wheat spikes cultured for 15 days, therefore it was not possible to distinguish between bleaching of spikelets due to fusarium isolate inoculation and bleaching due to natural senescence in spikes inoculated at 11 and 14 days in culture. Disease severity increased with the increasing concentration of *F. graminearum* conidia applied to wheat spikes. Depending on the isolates and days after heading in culture at which spikes were inoculated, inoculum concentrations of 10^3, 10^4, 10^5 and 10^6 macroconidia/mL led to 9-19%, 10-31%, 19 to 66 % and 38 to 87% disease severity, respectively, 7 days after inoculation (Figure 3.1). Analysis of variance (ANOVA) revealed that *F. graminearum* inoculum concentration significantly ($p< 0.01$) affects disease severity whereas the number of days after heading at which inoculum is administered doesn't affect disease severity significantly. Results indicated that the macroconidia concentration of 10^5 macroconidia/mL is best for screening fusarium head blight as disease severity at 10^3 and 10^4 macroconidia/mL were very low which may overestimate the moderate levels of resistance whereas excessive levels of disease obtained at 10^6 macroconidia/mL may overwhelm moderate levels of resistance. Moreover, types of isolates significantly ($p < 0.01$) affected disease severity only at 10^5 macroconidia/mL concentration. In the spike culture system, M9-07-1 was found to be the most virulent strain followed by M7-07-1, M1-07-2 and FGSC-9075.

Differences were detected in resistance to FHB between AC Nanda, Sumai-3 and 134 mutant lines. Sixty-five mutant lines had disease severity between 0 to 35% whereas 47 lines had disease severity between 54-100% (Figure 3.2). For AC Nanda disease severity was found to be 54% whereas Sumai-3 had disease severity around 35%. Significant phenotypic differences were observed between resistant and

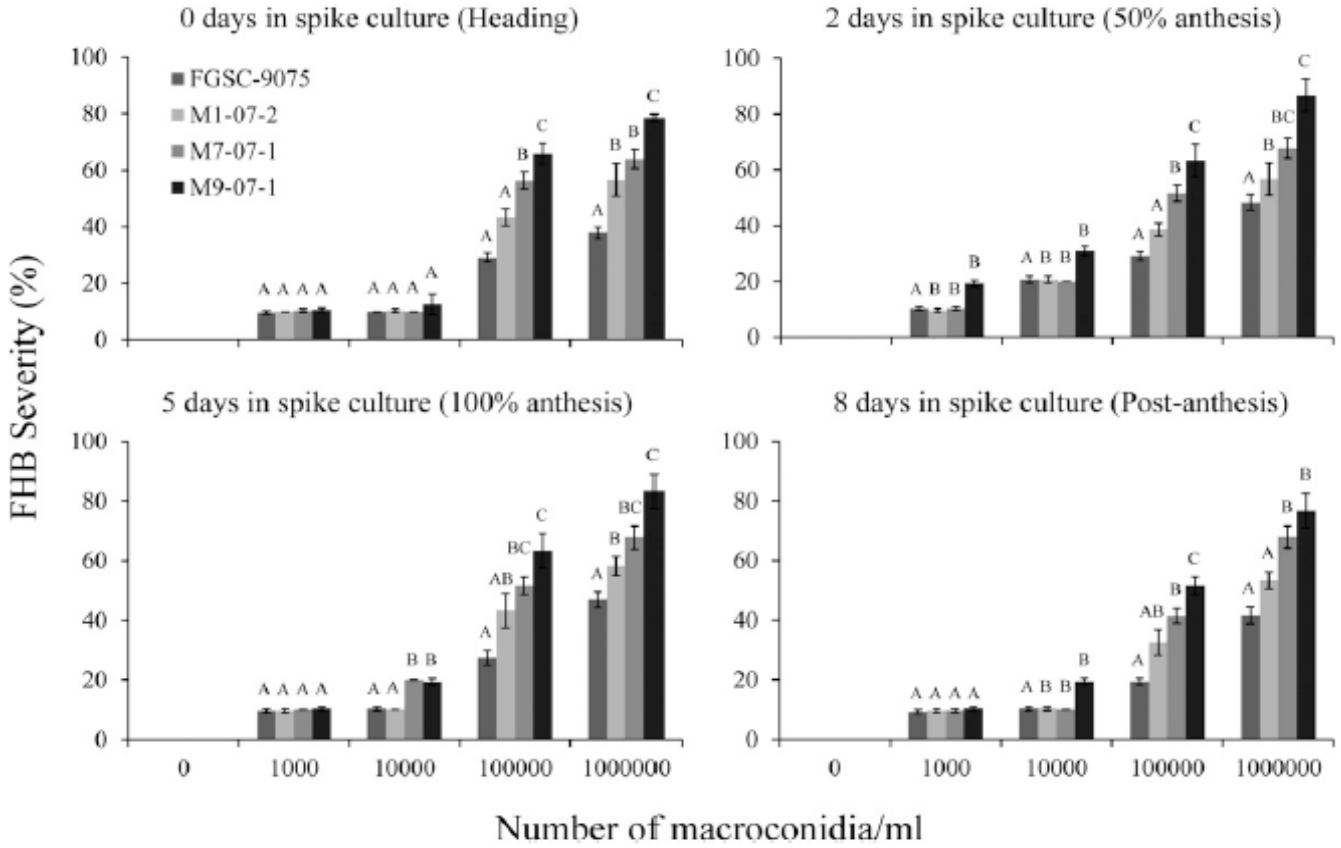


Figure 3.1: Effect of increasing concentrations of *Fusarium graminearum* isolates FGSC-9075 (■), M1-07-2 (■), M7-07-1 (■), and M9-07-1 (■) on Fusarium head blight disease severity in wheat cultivar AC Nanda spikes cultured for 0, 2, 5, and 8 days. Values are means ± standard deviations based on three independent determinations. Values followed by different letters show significant differences.

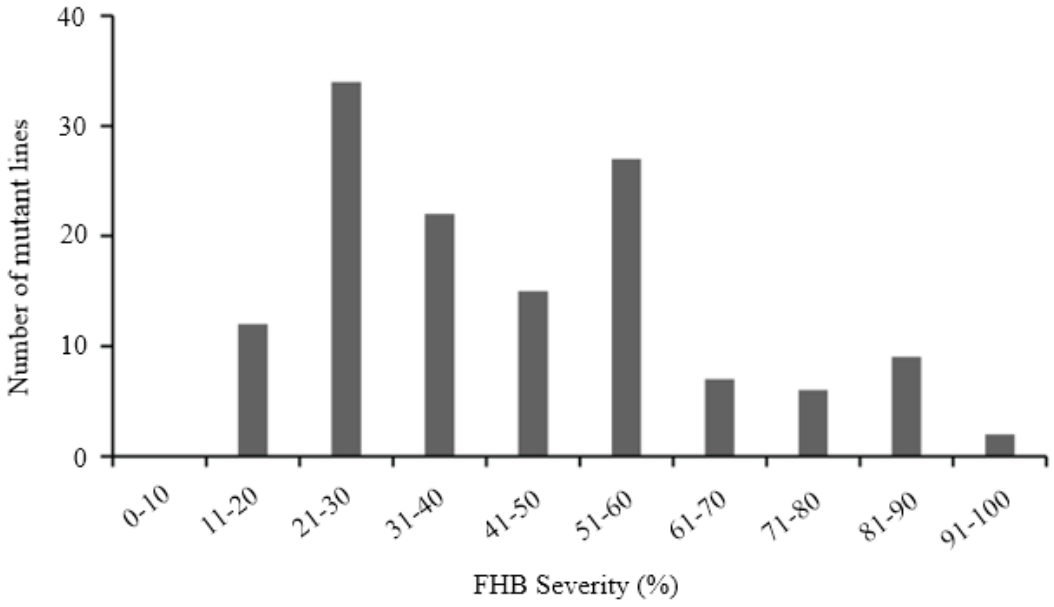


Figure 3.2: Frequency distribution of 134 wheat spike culture-derived variants (SCDV) indicating the range of Fusarium head blight severity.

susceptible wheat genotypes using the immature spike culture method has been observed in this study (Figure 3.3). The pairwise t-test revealed the statistically significant difference of FHB severity between AC Nanda and Sumai-3, with P value < 0.01 (Figure 3.4).

3.4.2 Identification of Single nucleotide polymorphism in *TaUGT*

Primer (*TaUGT2BF1/2R2*) designed to amplify *TaUGT-2* (GenBank Acc: EU568801.1) produced product in *A. speltoides* (putative B genome in hexaploid wheat) and AC Nanda. No PCR products were obtained with *T. monococcum* (A genome), and *A. tauschii* (D genome) and AC Nanda (2ABD) indicating the B genome specificity of the primer (Figure 3.5). DNA sequencing revealed only one change in nucleotide between AC Nanda (A) and Sumai (G) at position 450 bp from the translation initiation site in exon I (Figure 3.6). Similary, for *TaUGT-3* (*TaUGT3F1/3R2*), a PCR product was observed only in AC Nanda and A. speltoides (Figure 3.5). At position 1558 bp from translation initiation site in exon II, single nucleotide change from G in AC Nanda to T in Sumai-3 was observed (Figure 3.6). Primers for *TaUGT-EST* however amplified a product of 455 bp length from *T. monococcum*, *A. speltoides*, *A. tauschii* as well as AC Nanda (Figure 3.5). Only the D genome UGT-EST nucleotide sequence matched 100% with AC Nanda nucleotide sequence. One nucleotide change was found at position 229 bp between AC Nanda (G) and Sumai-3 (A) sequence which leads to the incorporation of an early stop codon in Sumai-3 (Figure 3.6).

For mutation screening of EMS mutant lines, high resolution melt (HRM) analysis was performed. A nested PCR approach was used to amplify sequence specifically from the B genome of *TaUGT-2* and *TaUGT-3*. Analysis of the difference plots for the fluorescent signals detected individual plants that vary in nucleotide sequence. Two distinct groups, one including AC Nanda and other including Sumai-3, were observed in the difference plots for *TaUGT-2B* as well as *TaUGT-3B* gene (Figure 3.7). At 450 bp from the translation initiation site in *TaUGT-2B* gene, 34 EMS mutants had nucleotide A like AC Nanda whereas 100 EMS mutant lines had nucleotide G like Sumai-3. Similarly, for *TaUGT-3B*, 29 EMS mutant lines had nucleotide G at position 1558 bp as in AC Nanda whereas105 EMS mutant lines had T as in Sumai-3. To confirm these results, independent gel eluted PCR products were sequenced. Sequencing validated HRM results. The D genome specific primer of *TaUGT-EST* however

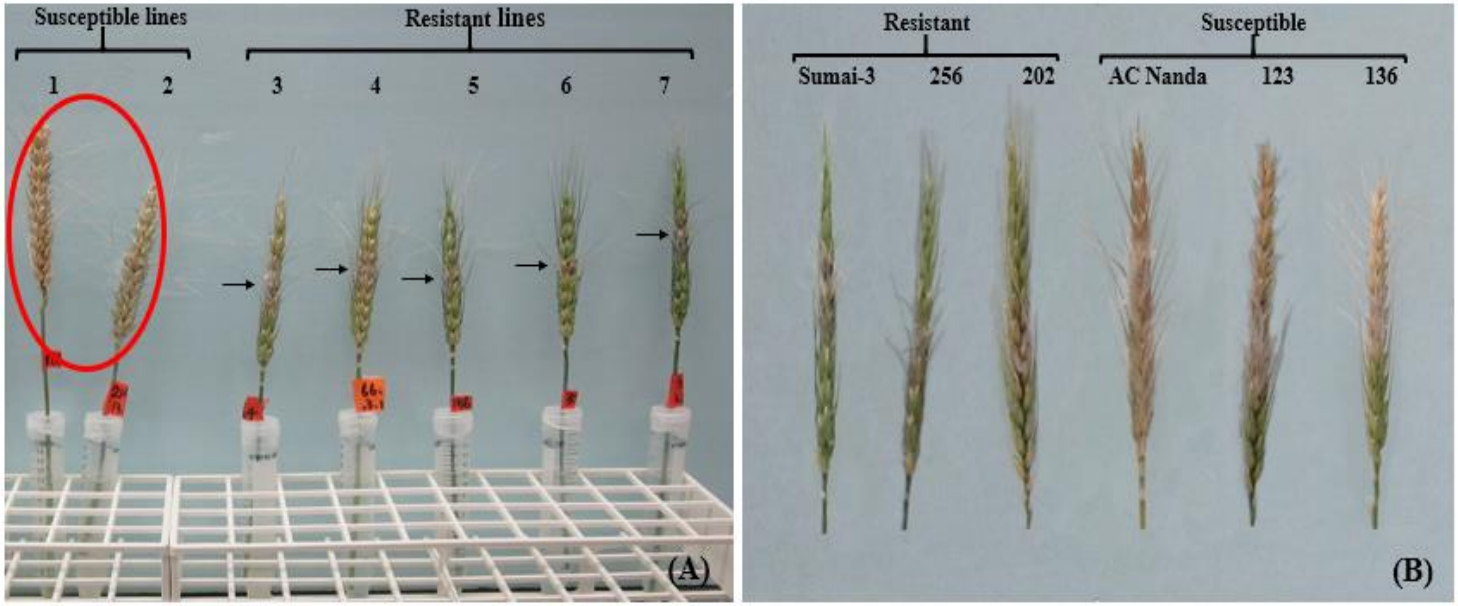


Figure 3.3 (A): The differentiation of resistant and susceptible immature spikes by using spike culture method. 1-2: susceptible lines; 3-7: resistant lines. The spikes within red circle pointed out were the susceptible wheat genotypes with bleached wheat spikes covered by fungal mycelium being point-inoculated by *F. graminearum* M7-07-1 strain. The black arrows point at the specific area showing necrotrophic or bleached symptoms caused by FHB disease. **(B):** This figure listed the resistant (FHB202, FHB256) and susceptible (FHB123, FHB136) lines used in this study for the determination of mycotoxins. Sumai-3 and AC Nanda are resistant and susceptible controls.

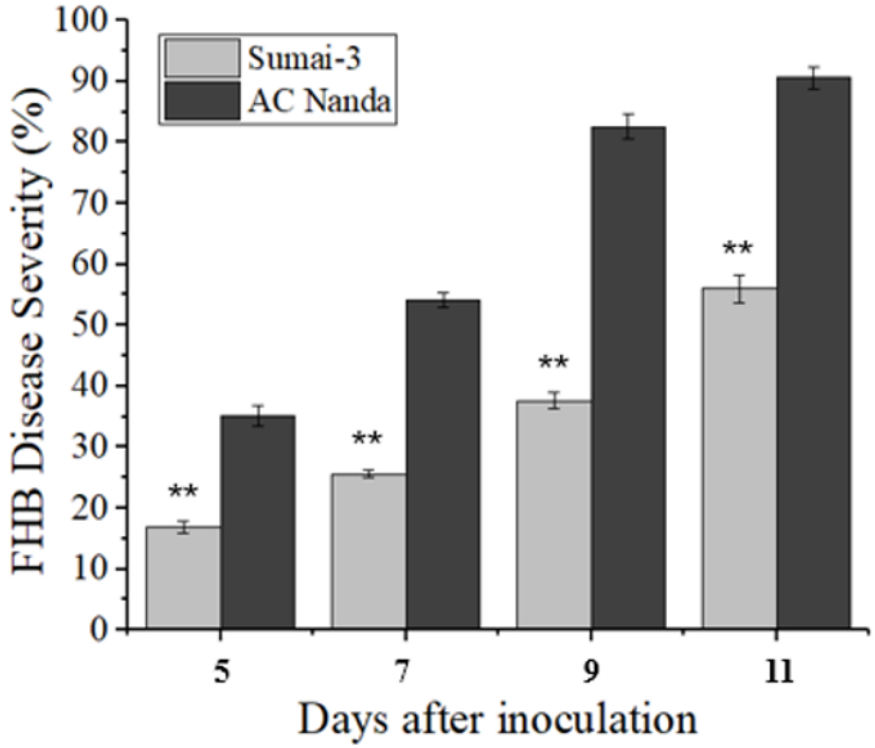


Figure 3.4: FHB disease severity of AC Nanda and Sumai-3 on 5, 7, 9, 11 days after inoculation by Fusarium isolate M7-07-1, based on the calculation of percentage of infected spikelets within a spike (Bai and Shaner, 1994). FHB disease severity = number of infected spikelets within a spike / number of total spikelets of the spike◊100%. ** shows the pairwise t-test results are significant at $P \leq 0.01$.

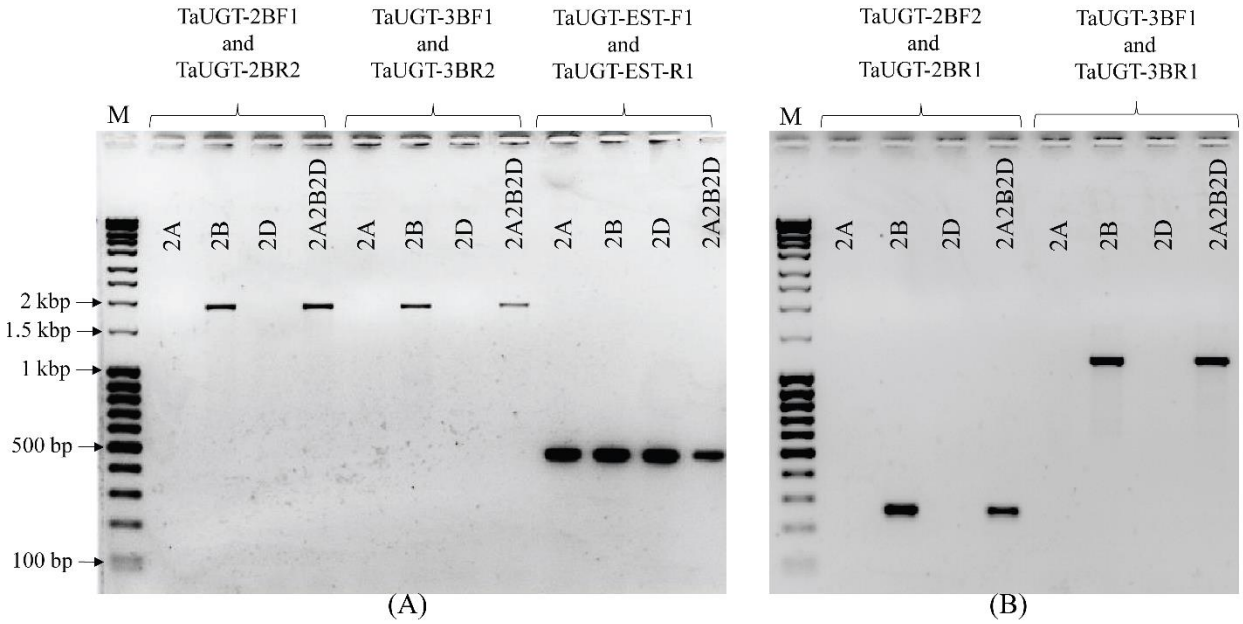


Figure 3.5: Genome specificity of the primers used in **A**, gene isolation and **B**, semiquantitative expression analysis. The electrophoretic profiles include the PCR products amplified from *Triticum monococcum* (2A), *Aegilops speltoides* (2B), *Aegilops tauschii* (2D), and Triticum aestivum 'AC Nanda' (2A2B2D).

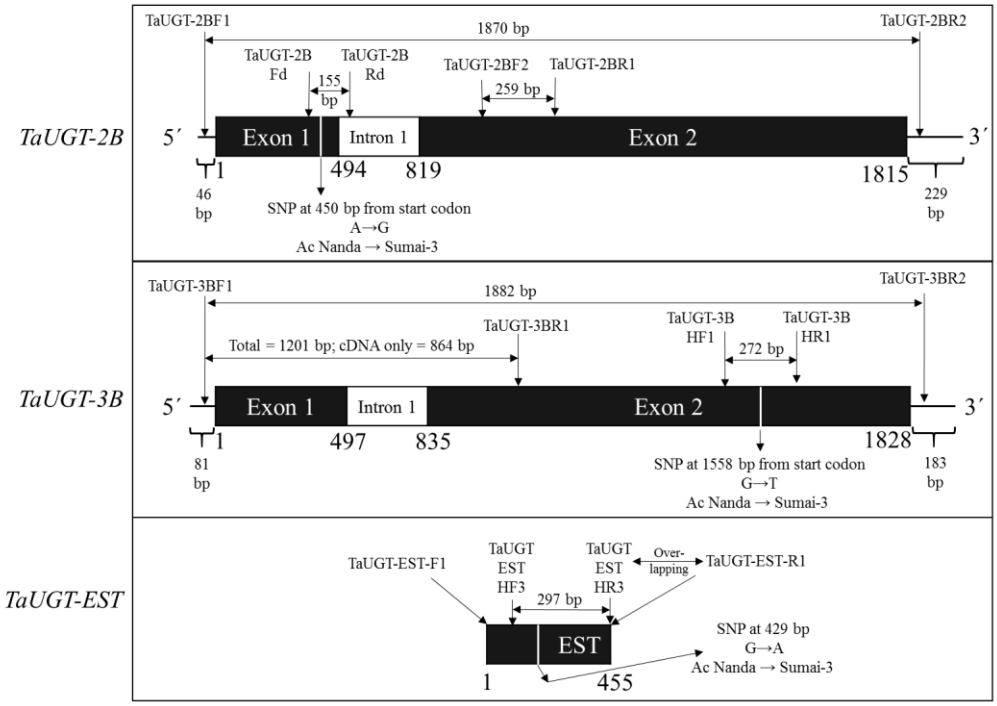


Figure 3.6: Schematic representation of *TaUGT-2B*, *TaUGT-3B*, and *TaUGT-EST* in wheat after comparing sequences from the present study to those available in NCBI. The sequences show primer sites, amplicon length, location of single nucleotide polymorphisms (SNP), and distribution of exons/introns/untranslated regions.

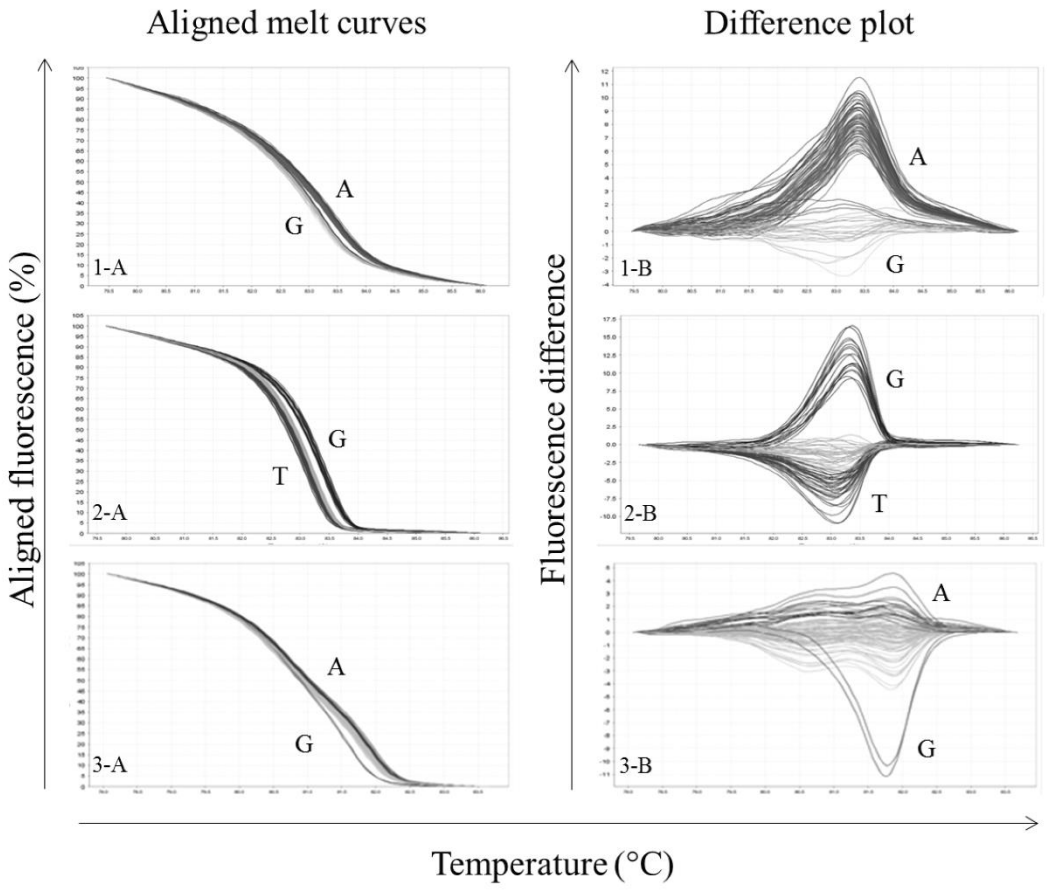


Figure 3.7: High resolution melt curve (HRM) analysis with primers *TaUGT-2B Fd/Rd-1*, *TaUGT-3BHF1/HR1*, and *TaUGT-EST HF3HR3* in terms of aligned fluorescence (F, %) versus temperature (T) curves, and ΔF/T difference curves separating Fusarium head blight resistant and susceptible genotypes into two distinct groups (1A and 1B and 2A and 2B). In 3A and 3B, only Sumai-3 was detected with single nucleotide polymorphisms.

produced only one cluster containing AC Nanda and all mutant lines. Only Sumai-3 differed as was expected due to the nucleotide difference at 229 bp (Figure 3.6). Significance level (TTEST) revealed a strong association ($p<0.01$) between the nucleotide variations at position 450 bp in *TaUGT-2* and 1558 bp in *TaUGT-3* with Fusarium head blight severity (Table 3.2). On the basis of nucleotide sequence variation and mean value of FHB disease severity at 7 and 9 days after inoculation, 15 of the most promising EMS mutant lines were selected that could be used in a breeding program.

3.5 Discussion

Evaluation of FHB resistance is complex and subject to environment (Parry et al., 1995). Since not all varieties and plants within the same variety flower simultaneously, some experimental variation between early and later flowering plants may exist. Moreover, final observation for this disease is taken after 21 days of inoculation which is time consuming. Therefore, one of the objectives of this study was to develop a rapid, uniform, reliable and laboratory-based method of fusarium head blight resistance screening of wheat. Cereal spike culture system previously developed (Ganeshan et al., 2010) was optimized to be used as screening tool for fusarium head blight resistance in wheat. Spikes of wheat cv. AC Nanda were point inoculated with 15 µL of 10^6, 10^5, 10^4 and 10^3 macroconidia/mL of four *F. graminearum* isolates FGSC-9075, M1-07-2, M7-07-1 and M9-07-1 after 0, 2, 5, 8, 11 and 14 days in culture (Figure 3.1). Disease severity increased with increasing concentration of *F. graminearum* conidia applied to wheat spikes. Analysis of variance (ANOVA) revealed that *F. graminearum* inoculum concentration significantly ($p < 0.01$) affects disease severity whereas the number of days after heading at which inoculum is administered doesn't affect disease severity significantly. Results indicated that a concentration of 10^5 macroconidia/mL is best for screening FHB, as disease severity at 10^3 and 10^4 macroconidia/mL were very low which may overestimate the moderate levels of resistance whereas excessive levels of disease obtained at 10^6 macroconidia/mL may overwhelm moderate levels of resistance. Moreover, isolate type significantly ($p < 0.01$) affected disease severity only at 10^5 macroconidia/mL concentration. In the spike culture system, M9-07-1 was found to be the most virulent strain followed by M7-07-1, M1-07-2 and FGSC-9075 which is consistent with the earlier report showing that the 3-ADON chemotypes are more

Table 3.2: One-way analysis of variance indicating significant effects of single nucleotide polymorphisms in B-genome specific *UDP-glucosyltransferases* on Fusarium head blight (FHB) disease severity.

Gene	Nucleotide position	Allele	Number of variant lines	Mean sum of squares for FHB severity	Significant at P
TaUGT-2B	450	A	34	6,129	≤ 0.001
		G	100		
TaUGT-3B	1,558	G	29	4,458	≤ 0.001
		T	105		

aggressive than the 15-ADON chemotypes based on disease development and DON production (Puri and Zhong, 2010). Further, screening for FHB resistance using spike culture could adequately differentiate FHB resistant Sumai-3 and FHB susceptible AC Nanda suggesting that this protocol can be used for routine screening of FHB resistance.

3.5.1 SCDV provides new sources of FHB resistance

Some FHB resistant sources such as Sumai-3 are known; however, their use in breeding programs is limited due to poor adaptability to the local environment, inferior agronomic traits and low grain quality (McMullen et al., 2012). The SCDV derived from AC Nanda (Canada western soft white spring wheat) with high FHB resistance may overcome these difficulties and can be incorporated into breeding programs. The SCDV population (134 wheat variants) differed in FHB resistance, ranging from 15 to 100%. Compared to Sumai-3, higher FHB resistance occurred in some SCDV and may be utilized as new sources of FHB resistance. Although no abnormal agronomic characteristic was observed while growing FHB resistant lines in the greenhouse, their performance needs to be examined in field disease nurseries at multiple sites and over multiple years.

3.5.2 *UDP-glucosyltransferase* SNPs associated with FHB resistance and reduced DON accumulation

To develop new FHB resistant cultivars, an understanding of the molecular mechanisms underlying FHB resistance is needed. The SNP detected in *TaUGT-2B* and *TaUGT-3B* were associated with FHB resistance in the SCDV population. The DON accumulation at various stages after *F. graminearum* inoculation in FHB resistant and susceptible wheat genotypes confirmed the association with *TaUGT-2B* and *TaUGT-3B*, as well as with FHB resistance. Although higher DON accumulation was observed at two DAI in FHB resistant genotypes compared to susceptible genotypes, induction of *UGT* expression appears to limit further DON accumulation. Therefore, a gradual decrease in DON concentration was observed after two DAI in FHB resistant wheat genotypes, which was associated with reduced FHB disease severity (Type II resistance). Most of the studies to-date have indicated induced *UGT* expression when plants were exposed to DON externally (Li et al., 2015). The results of our study support this as we observed differences in the in vivo DON accumulation pattern between FHB resistant and susceptible wheat genotypes. *UGT* transcript abundance was similar in all

wheat genotypes immediately after inoculation (0 DAI), irrespective of FHB disease resistance. The DON may act as an inducer for *TaUGT-2B* and *TaUGT-3B* in wheat; hence in FHB resistant genotypes, higher DON accumulation was observed that induced *UGT* during the initial stages of disease development. In FHB susceptible genotypes, low concentration of DON could not induce *UGT* expression during the initial stages of disease induction. However, as the disease progressed both DON concentration and FHB expression increased gradually (Sharma et al., 2018). Maximum UGT expression at six or eight DAI, in FHB susceptible genotypes, could not restrict DON accumulation, which resulted in higher FHB disease severity. Expression of *TaUGT-2B* and *TaUGT-3B* clearly differentiated FHB resistant from susceptible wheat genotypes. *TaUGT-EST* expression was also associated with FHB disease resistance except in SCDV 123.4. The induced expression of *UGT* and their association with FHB resistance and DON accumulation concurs with previous studies (Li et al., 2015; Lin et al., 2008; Lulin et al., 2010). The present study also identified the participation of B-genome specific *TaUGT-2* and *Ta-UGT-3* in FHB resistance. Based on previous studies, *Fhb1* confers Type II FHB resistance, mainly by regulating cell wall thickening (Gunnaiah et al., 2012) or DON detoxification (Kluger et al., 2015; Lemmens et al., 2005). Combined genetic and biochemical evidence suggest that Fhb1 includes a gene-for-*UDP-glucosyl transferase* (*UGT*) that conjugates DON to D3G or a gene that regulates *UGT* (Lemmens et al., 2005). A recent study reported a pore-forming toxin-like (PFT) gene at the *Fhb1* locus that conferred enhanced FHB resistance in wheat (Rawat et al., 2016), and concluded that DON detoxifification is independent of the PFT gene. The *UDP-glycosyltransferase* (*UGT*) genes were suggested to be located in the vicinity of the *Fhb1* locus contributing to FHB resistance by rapid DON detoxification to DON glycoside (Rawat et al., 2016). Consequently, several *UDP-glycosyltransferase* (*UGT*) genes induced by FHB inoculation or DON treatment were differentially expressed in cultivars varying in FHB resistance (Li et al., 2015; Lin et al., 2008; Lulin et al., 2010). In *F. graminearum*- infected young spikes of bread wheat (*Triticum aestivum* L.), expression of *TaUGT2* was induced, whereas expression of *TaUGT1* was repressed suggesting that *TaUGT2* may participate in wheat resistance to FHB (Lin et al., 2008). A DON-resistance related gene *TaUGT3* was also cloned and characterized from a FHB resistant wheat (variety Wangshuibai), overexpression of which improved DON tolerance in Arabidopsis (Lulin et al., 2010). Transgenic Arabidopsis (Shin et al., 2012) and wheat (Li et al., 2015) expressing a barley *UGT*,

provided a high level of resistance to FHB by detoxifying DON to D3G. It is important to note that DON is not the only virulence factor of *F. graminearum* (Paranidharan et al., 2008); therefore, it is important to characterize the chemotype of the isolate.

The SNP found in the present study are synonymous; however, they can induce a significant phenotypic effect due to their impact on gene function (Chagné et al., 2008; Kimchi-Sarfaty et al., 2007; Palatnik et al., 2003). These 'silent' substitutions may regulate translation speed, protein homeostasis, metabolic fate and sometimes post-translational modifications (Shabalina et al., 2013).

In conclusion, the present study established the validity of utilizing spike culture as a screening tool for FHB resistance. The spike culture method clearly differentiated FHB resistance of AC Nanda, Sumai-3 and 134 SCDV genotypes. The SNPs in *TaUGT-2B* and *TaUGT-3B* were associated with FHB resistance in wheat. The FHB resistant wheat variants identified can be utilized as new genetic resources in wheat breeding programs. The SNP present in *TaUGT-2B* and *TaUGT-3B* are currently being used for marker assisted selection (MAS) for FHB resistance in wheat (unpublished results). The HRM assay was also established as a tool to identify SNP. FHB resistance is a quantitative trait; the FHB resistant SCDV genotypes can be used to identify and characterize candidate genes in wheat to improve FHB resistance.

CHAPTER 4. WHEAT *IN VITRO* SPIKE CULTURE SCREENING OF FUSARIUM HEAD BLIGHT INFECTION REVEALED A POSITIVE CORRELATION BETWEEN DISEASE SEVERITY AND MYCOTOXIN ACCUMULATION DETERMINED USING LC-MS/MS

Study 2

The main objective of this study was to optimize a LC-MS/MS method to determine the mycotoxin concentrations in wheat spikes and correlate it to FHB disease severity.

CH conducted the experients described in the manuscript.

Huang, C, Gangola, M.P. and Chibbar, R.N. (2019) Wheat *in vitro* spike culture screening of Fusarium Head Blight infection revealed a positive correlation between disease severity and mycotoxin accumulation determined using LC-MS/MS. Canadian J Plant Pathology (submitted after revision).

4.1 Abstract

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe is a major disease of wheat (*Triticum aestivum* L.). FHB preferentially infects immature spikes and accumulates mycotoxin in developing grains, reducing yield and nutritional quality. Mycotoxins in grains adversely affect human health at concentrations >2 mg kg^{-1}. The mycotoxin are type B trichothecene group of compounds including deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and nivalenol (NIV) that also act as virulence factors promoting fungal growth during FHB infection and disease progression. An enzyme UDP-glucosyl transferase (EC 2.4.1.x) glycosylates DON to deoxynivalenol-3-glucoside (D3G), thus rendering it ineffective as a virulence factor. The present study analyzed the association of DON, 3A-DON and D3G with FHB severity using *in vitro* spike culture for FHB screening. The mycotoxin in FHB resistant and susceptible spikes were extracted using a single step extraction method and their concentration was determined using a LC-MS/MS method that differentiated all the five mycotoxin. Pairwise comparisons of FHB resistant and susceptible SCDV lines using Tukey's method showed significant ($P \leq 0.001$) variation for disease severity and accumulation of DON, 3-ADON, and D3G at seven and nine days after fungal inoculation. FHB severity showed significant positive correlation to accumulation of DON, 3-ADON and D3G, but an inverse correlation to the ratio between D3G and DON.

4.2 Introduction

Fusarium head blight (FHB), also called scab, is an extremely detrimental disease in cereals such as wheat, barley, corn and oat, especially in humid climates in temperate regions (Wegulo, 2012). FHB is predominantly caused by *Fusarium graminearum* Schwabe [telomorph: *Gibberella zeae* (Schw.) Petch]. However, *F. pseudograminearum*, *F. culmorum*, *F. avenaceum* and *F. cerealis* are some of the other species that also cause FHB (Desjardins and Proctor, 2007; Logrieco and Moretti, 2008). FHB infects immature wheat spikes and restricts grain development causing up to a 50% reduction in grain yield in North America (McMullen et al., 1997; Goswami and Kistler, 2005). Fusarium infection produces mycotoxins of tricothecene group that, (i) act as virulence factors for the pathogen by inhibiting protein synthesis in plants (Desjardins, 2006; Proctor et al., 2008; Amarasinghe et al., 2015), (ii) are phytotoxic and cause plants to develop non-specific disease symptoms such as chlorosis, necrosis, and

wilting (Desjardins, 2006; Nishiuchi et al., 2006), and (iii) in humans negatively affect the integrity, and immunity of the gastrointestinal tract and reduce the absorption of nutrients from food (Liew and Mohd-Redzwan, 2018).

The type B trichothecene mycotoxins deoxynivalenol (DON), nivalenol (NIV) and their acetylated products are the major mycotoxins present in Fusarium infected wheat spikes (Figure 2.2) (Mallmann et al., 2017). In Canada, DON is the major mycotoxin detected in wheat kernels infected by the *Fusarium graminearum*, *F. culmorum*, and *F. cerealis* isolates (McMullen et al., 1997; Amarasinghe et al., 2015). In Asia and Europe both DON and NIV are predominant (Ichinoe et al., 1983). The *F. graminearum* isolates also produce the acetylated DON derivatives, 3-Acetyldeoxynivalenol (3-ADON) and 15-Acetyldeoxynivalenol (15-ADON). The 3-ADON producing *F. graminearum* isolates are the most virulent compared to 15-ADON and NIV producing isolates (Amarasinghe et al., 2013). The 3-ADON producing isolates have migrated from eastern to western Canada and have become predominant, replacing 15-ADON producing isolates (Ward et al., 2008). Although NIV is less toxic to plants compared to DON, it has more severe toxic effects in animals and humans (Schothorst and Van Egmond, 2004; Cheat et al., 2015). In plants, DON, by an enzymatic reaction catalyzed by *UDP-glucosyltranferases* (EC 2.4.1.x), can be converted into deoxynivalenol-3-glucoside (D3G) (Poppenberger et al., 2003). D3G is a modified mycotoxin produced by plants and has been referred as masked mycotoxin according to International Life Sciences Institute (ILSI, 2011). Initially, D3G was considered a non-toxic form of DON, but later it was shown that animal gut microflora can reconvert D3G to DON (Poppenberger et al., 2003; Lemmens et al., 2005; Berthiller et al., 2013; Kluger et al., 2015). Therefore, it is important to determine the D3G concentration during FHB progression and in infected grains.

Correlation between mycotoxin accumulation and FHB severity is still debated, as no (Ji et al., 2015), and moderate to strong ($r = 0.315$-0.610; Miedaner et al., 2016; Huang et al., 2018) positive correlations have been reported in previous studies using liquid chromatography with mass spectrometry, Enzyme-Linked Immunoassay (ELISA) assay, and gas chromatography with mass spectrometry, respectively. Similarly, the association of D3G to DON ratio to FHB resistance is not well established. Nakagawa et al. (2017) found no relation between D3G to DON ratio and FHB resistance however, the ratio was lower in FHB susceptible genotypes compared to moderately FHB resistant spring wheat varieties (Amarasinghe et al., 2016). In a previous study using *in*

vitro immature spike culture to screen for FHB resistance, combined with ELISA to determine DON concentration, a positive correlation to FHB severity was detected (Sharma et al., 2018). However, simultaneous analysis of all mycotoxins and D3G using ELISA is not feasible whereas liquid chromatography coupled with mass spectroscopy (LC-MS/MS) has been widely utilized to determine mycotoxins and D3G concentrations simultaneously (Habler et al., 2017; Kokkonen and Jestoi, 2009; Yoshinari et al., 2012; Zuo et al., 2018) (Table 4.1). Therefore, to evaluate the association of FHB severity with mycotoxin accumulation or D3G to DON ratio, selected spike culture derived variants (SCDV) were screened for FHB severity using *in vitro* spike culture and mycotoxin concentration in spikes was determined using a LC-MS/MS method.

4.3 Materials and methods

4.3.1 Standards and chemicals

Standards of DON, 3-ADON, D3G, 15-ADON, and NIV were purchased from Romer Labs (Washington, MO, USA) whereas LC-MS grade water, LC-MS grade methanol, acetonitrile, acetic acid, and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.3.2 Plant material and evaluation of FHB severity

Wheat (*Triticum aestivum* L.) landrace Sumai-3 and cv AC Nanda (Sadasivaiah et al., 2000) were used as FHB resistant and susceptible genotypes, respectively to systematically evaluate FHB resistance using the recently described immature spike culture screening technique (Sharma et al., 2018). In addition, two FHB resistant (FHB202 and FHB256) and susceptible (123 and 136) wheat genotypes identified from a population of ethyl methane sulfonate treated spike culture-derived variants were also evaluated (Ganeshan and Chibbar, 2017; Sharma et al., 2018). The M_3 generation seeds of all the genotypes were grown in a greenhouse (University of Saskatchewan, Saskatoon SK Canada) with 16/8 h of day/night cycle (350 µmol m^{-2} s^{-1}) and average daily temperature of 27.3/20.6°C during May-July 2016, respectively. The completely emerged immature spikes were collected, placed in spike culture media and point inoculated with M7-07-1 (moderately virulent) isolate of *Fusarium graminearum* (Sharma et al., 2018).

The percentage of symptomatic spikelets in a spike (PSS) was used to assess

Table 4.1: Comparison of the optimized method with other LC-MS/MS based methods to determine mycotoxin concentrations.

Parameters	Present method	Kokkonen & Jestoi (2009)	Yoshinari et al. (2012)	Habler et al. (2017)	Zuo et al. (2018)
Sample preparation	One step (no cleanup or drying)	Accelerated Solvent Extractor	Extraction, column cleanup and drying	Extraction, column cleanup and drying	Extraction, column cleanup and drying
Sample weight used	0.6 g of wheat meal	10 g of wheat, barley and oat meal	25 g of corn meal	5 mL of degassed beer	5 g of wheat, corn and oat meal
Run time (min)	25	30	7	25	25
Compounds analyzed[a]	NIV, DON, D3G, 15-ADON and 3-ADON	NIV, DON and 28 other mycotoxins	DON, D3G, 15-ADON and 3-ADON	DON, D3G, 15-ADON, 3-ADON and 17 other mycotoxins	DON, 15-ADON and 3-ADON
R^{2}[b]	>0.99	-	-	>0.99	>0.99
LOD (ppb)[b]	0.3-0.75	25.0-241.7	0.5-2.7	1.2-6.9	1.0-6.0
LOQ (ppb)[b]	0.5-2.5	55.0-500.0	1.3-6.2	3.5-20	3.0-20.0
Repeatability (RSD%)[b]	2.2-4.8	5.0-8.7	3.4-7.4	1.0-4.0	8.1-9.1
IP (RSD%)[b]	1.6-3.3	14.0-16.0	-	2.0-7.0	-
R_E (%)[b]	82.1-90.5	77.0-91.4	105.2-121.4	74.3-105.7	82.7-87.1
R_M (%)[b]	83.9-91.4	9.4-45.0	-	-	-

FHB severity (%) per spike = (number of symptomatic spikelets × 100) / total number of spikelets (Bai and Shaner, 1994). The visual rating scale for FHB severity was also determined as described by Stack and McMullen (1995). The spikes in three replications were collected at 2, 5, 7 and 9 days after inoculation and stored at -80°C till further analysis. These FHB infected spikes from FHB resistant and susceptible wheat genotypes were utilized for mycotoxin extraction and determination.

4.3.3 Mycotoxin extraction

The FHB infected wheat spikes (three replicates) were used for mycotoxin extraction. Each frozen spike was ground separately using a pestle and mortar. The ground sample from three spikes (~2.0 g) was pooled and mixed thoroughly (1 min) by a vortex mixer. The ground powder was freeze dried (Freezone 18, Labconco Corporation, Kansas City, Missouri, USA). From the pooled freeze dried sample, three replicates (0.6 g each) were separately mixed with 4 mL of extraction buffer (Acetonitrile:water; 84:16, v/v) in a 15 mL disposable tube. The solution was mixed for 30 s using a vortex mixer and kept on a rotary shaker (Barnstead, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 180 rpm for 60 min. Thereafter, the solution was kept at room temperature for 20 min. The supernatant (1 mL) was filtered through a 0.2 µm nylon filter (Fisher Scientific, Fair Lawn, NJ, USA) attached with 1 mL syringe. The filtrate was diluted 10 times with 9:1 solution of mobile phase A [LC grade water and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate] and B [LC grade methanol and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate] to bring the sample concentrations within the range of calibration curves.

4.3.4 LC-MS/MS method – instrumentation and analytical conditions

Mycotoxin analysis was performed on a 4000 QTRAP LC-MS/MS system (AB SCIEX, Framingham, MA, USA) that had a high-performance liquid chromatography with an electrospray ionization (ESI) and a hybrid triple quadrupole ion trap mass spectrometer. The analysis of chromatograms was performed using Analyst 1.6.2 software (AB SCIEX, Framingham, MA, USA). The auto-sampler was set at 4°C and 10 µL of injection volume whereas the chromatographic separations were achieved at 40°C using Zorbax Eclipse Plus C18 analytical

column (2.1×100 mm, Agilent Technologies, Santa Clara, CA, USA). The compounds were eluted using a gradient run of solvent A [LC grade water and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate] and B [LC grade methanol and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate] at 300 μL min^{-1} flowrate till 25 min (Table 4.2). The negative electrospray ionization (ESI-) mode was applied for the first 12 min (period 1) of mass spectrometer analysis, and thereafter, both positive and negative modes were applied (period 2). The operating conditions set for MS were as follow: during period 1, curtain gas (CUR) was set at 40 psi, ion voltage spray (IS) was set at -4500 V, the Turbo ion spray interface temperature (TEM) was set at 550°C, whereas nebulizer gas (GS1), heater gas (GS2), and CAD were set at 50, 70 and 6, respectively. Entrance potential (EP) was set at -10.00. During period 2, parameters were set as following: CUR: 40 psi, IS: -4500/5500 V (-ve/+ve mode), TEM: 550°C, GS1: 50, GS2: 70, EP: -10.00 and 10.00. The other parameters including collision energy (CE), collision exit potential (CXP) and declustering potential (DP) set for compounds were summarized as Table 4.3.

4.3.5 Assessment of the method

The optimized method was evaluated for its performance based on its coefficient of determination, limit of detection (LOD), limit of quantification (LOQ), recovery percentage, repeatability and intermediate precision. Calibration curves using serial dilutions (1.0, 2.5, 5, 10, 25, 50 and 100 ng mL^{-1} for DON and 3-ADON; 0.5, 1.25, 2.5, 5, 12.5, 25 and 50 ng mL^{-1} for D3G; 2.5, 6.25, 12.5, 25, 62.5, 125 and 250 ng mL^{-1} for NIV and 15-ADON) of the standards, were utilized to calculate regression equations and coefficients of determination for each mycotoxin. The LOD and LOQ for the compounds were determined with a signal-to-noise ratio of 3:1 and 5:1, respectively (ICH harmonized tripartite guideline, 2005).

The blank wheat matrix was spiked (six replicates each) with three concentrations (5, 50 and 100 ng mL^{-1}) of mycotoxins and then processed using the optimized extraction method followed by the determination of mycotoxin concentrations using the LC-MS/MS method. The recovery percentage (R%) was calculated as, R% = [(observed concentration×100)/actual concentration]. The effect of matrix or matrix-induced signal suppression/enhancement (SSE) was determined by comparing the signal intensity for standards in solvent and in wheat matrix spiked

Table 4.2: Gradient method of LC-MS/MS to analyze mycotoxins.

Step	Total Time (min)	Solvent A[a] (%)	Solvent B[b] (%)
1	0.00	90.0	10.0
2	0.50	90.0	10.0
3	15.00	0.0	100.0
4	15.50	90.0	10.0
5	25.00	90.0	10.0

[a]Solvent A: LC grade water and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate.

[b]Solvent B: LC grade methanol and acetic acid in a 499:1 (v/v) ratio with 5 mM ammonium acetate.

Table 4.3: LC-MS/MS parameters optimized to analyze mycotoxins.

Mycotoxins	Precursor Ion (*m/z*)[a]	Product Ions (*m/z*)[a] Quantifier Qualifier	Polarity	DP[b] (V)	Collision Energy (V)	CXP (V)[c]	Molecular weight
Nivalenol	371.1[M+CH_3COO]-	281.000	Negative	-60	-16	-7	312
		311.100		-60	-36	-7	
DON	355.1[M+CH_3COO]-	295.000	Negative	-55	-16	-9	296
		265.000		-55	-22	-7	
15-ADON	356.2[M+NH_4]+	321.200	Positive	46	19	20	338
		339.200		46	21	22	
3-ADON	397.2[M+CH_3COO]-	337.000	Negative	-55	-14	-11	338
		397.155		-55	-22	-7	
D3G	517.2[M+CH_3COO]-	457.200	Negative	-70	-20	-13	458
		427.100		-70	-30	-11	

[a](*m/z*) shows the mass to charge ratio. [b]DP = declustering potential. [c]CXP = collision exit potential.

with standards before extraction. The SSE was calculated as, SSE = [(peak area in wheat matrix/peak area in a solvent) ×100] using two concentrations of mycotoxins, 5 and 50 ng mL^{-1}. The known concentrations (5, 50 and 100 ng mL^{-1}) of standards alone and in spiked wheat matrix (six replicates each) were also evaluated on the same day (to determine repeatability) and on three different days (to calculate intermediate precision). Repeatability and intermediate precision were expressed as percent relative standard deviation (Gangola et al., 2014). In each run, six quality controls of three mycotoxins concentrations were also tested to assure the accuracy of this method. These controls included a wheat blank matrix fortified with known concentrations of mycotoxins. The controls were processed along with the samples and evaluated assuring method reliability in each run.

4.3.6 Statistical analysis

The multiple treatment comparisons for mycotoxin accumulation among FHB resistant and susceptible genotypes were performed using Tukey's method in SAS 9.4 (SAS, Cary, NC, USA).

4.4 Results

4.4.1 Assessment of LC-MS

The mycotoxins were extracted using a simple method as described by Ovando-Martínez et al. (2013) and Simsek et al. (2013) with some modifications. The sample weight and extraction buffer volume were reduced to 0.6 g and 4 mL in the present study, respectively. The optimized LC-MS/MS method eluted NIV (4.99 min), DON (9.13 min), D3G (9.80 min), 15-ADON (13.38 min), and 3-ADON (13.39 min) with in 25 min of total run time (Figure 4.1). Each sample in LC-MS/MS was run in two parts: (i) the first part was up to 12 min with negative mode only as all compounds (NIV, DON and D3G) eluted during that runtime were of negative polarity where as, (ii) the second part was performed from 12 to 25 min in both positive and negative modes as 15-ADON (Positive polarity) and 3-ADON (Negative polarity) eluted at the same retention time (13.4 min) therefore, running both modes was necessary for the analysis (Figure 4.1). Performance of the optimized method was further evaluated for distinct qualitative parameters including linearity, sensitivity, accuracy and precision.

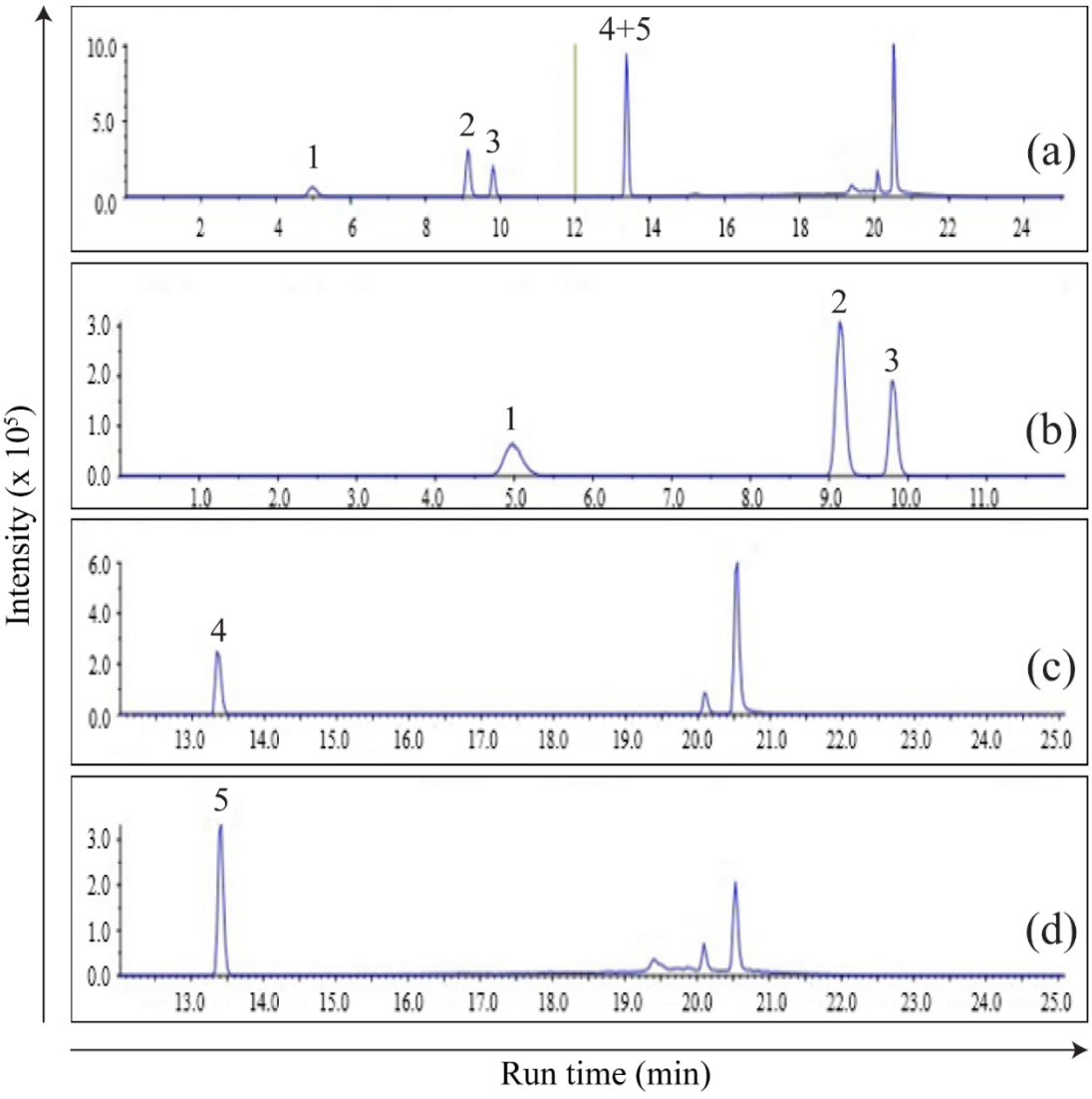


Figure 4.1: Mycotoxin determination using the optimized LC-MS/MS based gradient approach, **(a)** Chromatogram showing separation of nivalenol (1) (NIV; -ve polarity), deoxynivalenol (2) (DON; -ve polarity), deoxynivalenol-3-glucoside (3) (D3G; -ve polarity), 15-acetyldeoxynivalenol (4) (15-ADON; +ve polarity), and 3-acetyldeoxynivalenol (5) (3-ADON; -ve polarity) in a wheat matrix-based standards mix. The unlabeled peaks between 19 and 21 min of run time were from wheat matrix. **(b)** Chromatogram showing a run up to 12 min in negative electrospray ionization (ESI-) mode and separating NIV, DON, and D3G as having similar polarity. **(c)** Chromatogram showing run from 12 to 25 min in ESI- mode to analyze 3-ADON. **(d)** Chromatogram showing run from 12 to 25 min in ESI+ mode to analyze 15-ADON.

The optimized method was first evaluated by calculating coefficient of determination (R^2) and regression line equations from calibration curves of all the standards that were utilized to determine mycotoxin concentrations in wheat spikes (Table 4.4). The R^2 values for all the compounds were >0.99. Limit of detection (LOD) and limit of quantification (LOQ) were the lowest for D3G (0.3 and 0.5 ng mL^{-1}) followed by DON/3-ADON (0.5 and 1.0 ng mL^{-1}) and NIV/15-ADON (1.5/2.5 ng mL^{-1}), respectively. Recovery percent for NIV, DON, D3G, 15-ADON and 3-ADON were 85.0, 84.7, 90.0, 90.5 and 82.1%, respectively. The effect of wheat matrix was also examined for the optimized method by calculating percentage of signal suppression or enhancement (SSE) which were 85.8, 86.5, 91.4, 88.3 and 83.9% for NIV, DON, D3G, 15-ADON and 3-ADON, respectively. Repeatability and intermediate precision were determined as relative standard deviation percentage (RSD%). The optimized method showed variation in RSD% from 2.2 to 4.8% and from 1.6 to 3.3% for repeatability and intermediate precision of the mycotoxin studied, respectively (Table 4.4).

4.4.2 Comparing FHB resistant and susceptible genotypes using the optimized LC-MS/MS method

The optimized method was validated by determining the mycotoxin concentrations in FHB infected wheat spikes at different days after pathogen inoculation using the selected SCDV lines (Figure 4.2). The fungal isolate M7-07-1 used in the present study mainly accumulated DON, 3-ADON and D3G in the infected spikes. Other mycotoxins including NIV and 15-ADON were either not detected or present only in trace amounts. The FHB resistant genotypes (FHB202, FHB256 and Sumai-3) contained significantly ($P \leq 0.001$) lower concentrations of DON (0.2-0.3, 3.4-4.8, 3.0-5.9, and 2.4-6.5 mg kg^{-1}), D3G (0.17-0.23, 1.7-2.5, 2.4-3.2, and 2.4-4.9 mg kg^{-1}) and 3-ADON (0.4-0.6, 1.1-2.7, 0.9-2.9, and 1.3-2.7 mg kg^{-1}) in the spikes at all the stages after inoculation (2, 5, 7 and 9 days after inoculation) compared to that of DON (0.3-0.8, 7.1-11.5, 13.0-17.8, and 15.0-20.1 mg kg^{-1}), D3G (0.2-0.5, 3.5-3.9, 3.7-7.6, and 8.6-13.3 mg kg^{-1}) and 3-ADON (0.9-1.4, 4.8-6.3, 4.3-5.6, and 5.2-6.6 mg kg^{-1}) concentrations in FHB susceptible genotypes (123, 136, AC Nanda), respectively (Figure 4.2).

Table 4.4: Assessment of LC-MS/MS based method for linearity, sensitivity, accuracy and precision.

Compounds[a]	t_R[b] (min)	R^2[b]	LOD[b] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	Recovery (%) using different concentrations (ng mL^{-1})			Effect of matrix using different concentrations (ng mL^{-1})		Repeatability/IP[b] (% relative standard deviation) using concentrations (ng mL^{-1})		
					5	50	100	5	50	5	50	100
NIV	4.99	0.999	0.75	2.5	87.7	84.0	83.4	86.2	85.5	1.2/2.0	2.4/1.3	2.9/3.7
DON	9.13	0.999	0.50	1.0	88.1	85.8	80.2	82.5	90.5	2.1/2.2	1.9/2.6	3.1/1.5
D3G	9.80	0.999	0.30	0.5	95.6	88.6	85.9	93.5	89.3	4.2/3.1	5.6/3.8	1.0/3.1
15-ADON	13.38	0.999	0.75	2.5	89.8	90.5	91.2	86.0	90.6	4.2/1.1	5.6/2.7	4.8/1.0
3-ADON	13.39	0.999	0.50	1.0	80.2	84.6	81.6	80.6	87.2	2.7/1.0	4.0/3.1	1.7/3.1

[a]NIV, DON, D3G, 15-ADON and 3-ADON represent nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, 15-Acetyldeoxynivalenol, and 3-Acetyldeoxynivalenol, respectively. [b]t_R, R^2, LOD, LOQ and IP stand for retention time, coefficient of determination, limit of detection, limit of quantification, and intermediate precision, respectively.

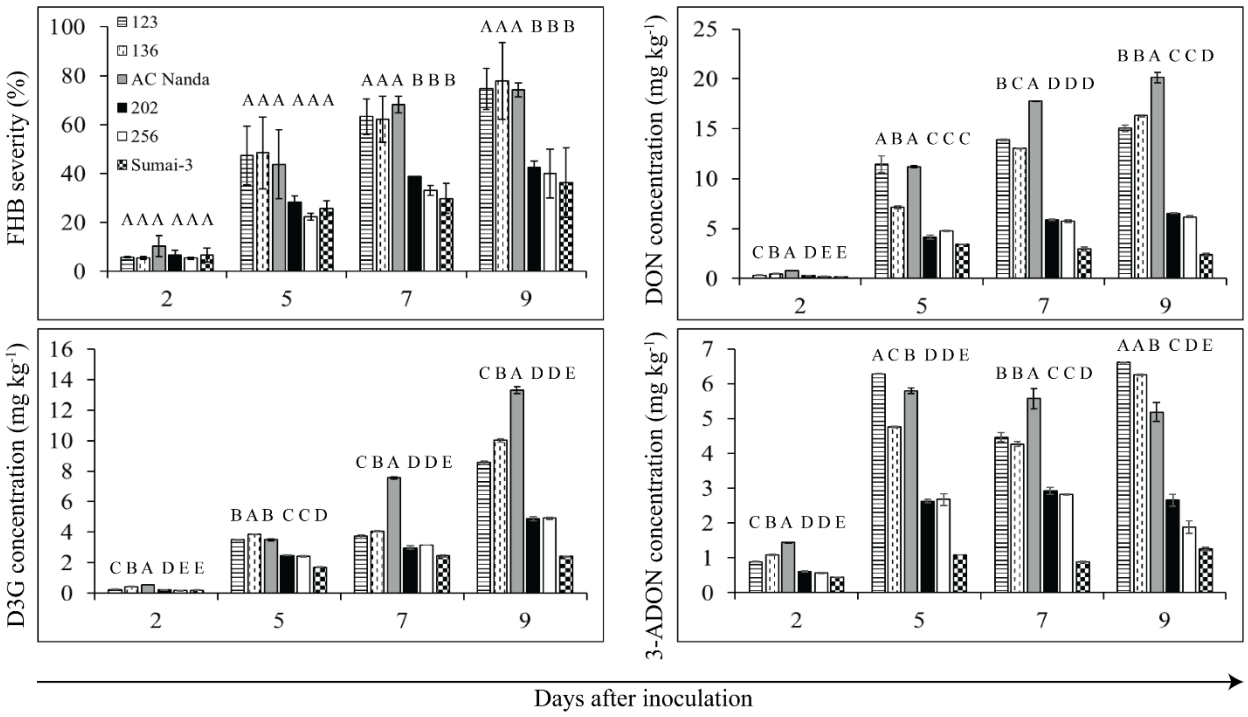


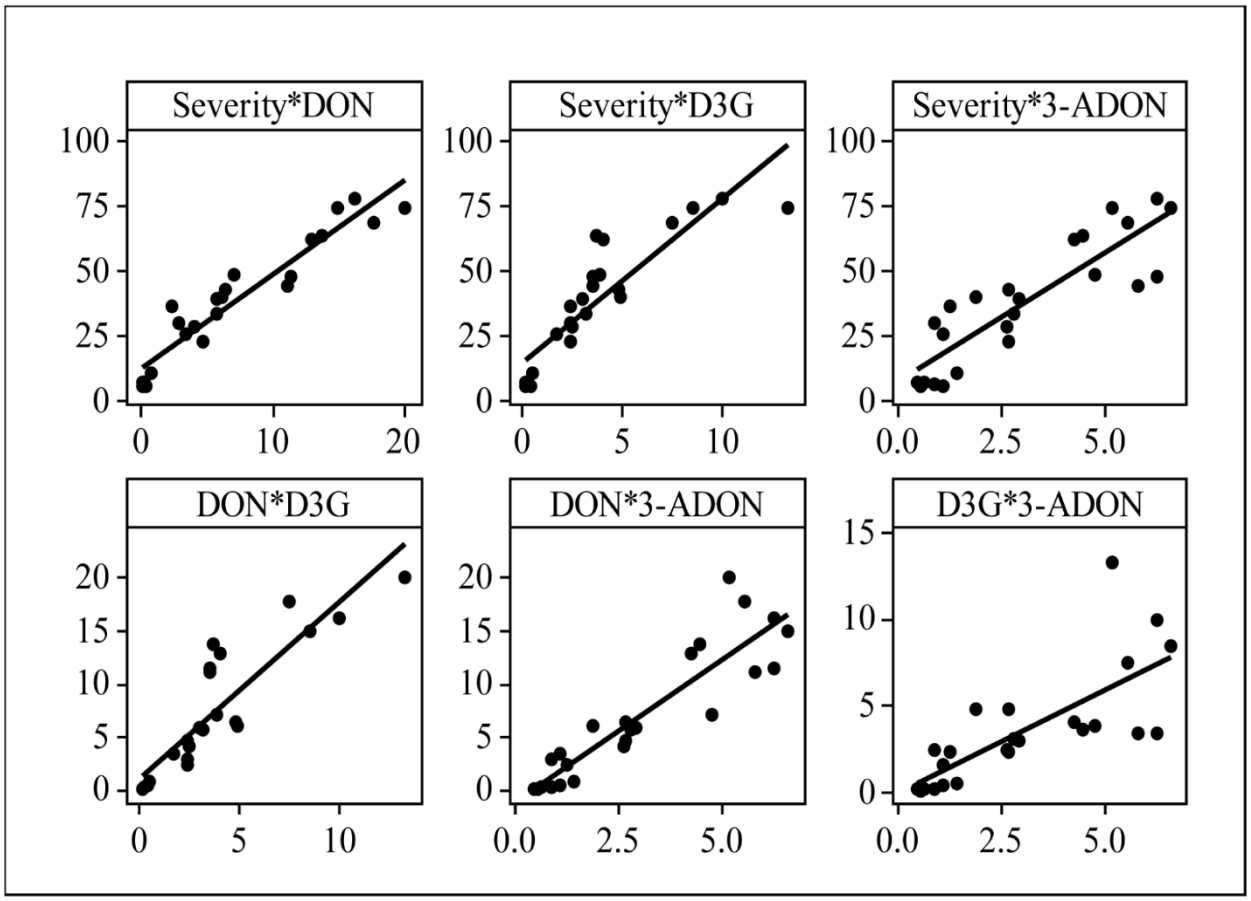
Figure 4.2: Multiple comparisons using Tukey's method among FHB -resistant (FHB202, FHB256 and Sumai-3) and -susceptible (123, 136 and AC Nanda) wheat genotypes for FHB severity (%) and accumulation of mycotoxins [deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), and 3-Acetyldeoxynivalenol (3-ADON)] at 2, 5, 7 and 9 days after fungal inoculation. Significant ($P < 0.001$) differences among values are represented by different letters in the bar-graphs.

4.4.3 Correlation between FHB severity and mycotoxin accumulation

FHB severity (PSS) did not vary significantly between FHB -resistant (5.4-6.7 and 22.4-28.2%) and -susceptible (5.4-10.4 and 43.8-48.4%) genotypes at two and five DAI, respectively (Figure 4.2). However, FHB susceptible (62.2-68.3% and 74.1-77.8%) and resistant (29.8-38.9% and 36.3-42.4%) genotypes had significant ($P \leq 0.001$) difference for disease severity at seven and nine days after fungal inoculation (Figure 4.2). FHB severity using a visual scale (Stack and McMullen, 1995) also showed a significant ($P \leq 0.01$) difference between AC Nanda and Sumai-3 at various stages after fungal inoculation thus confirming the disease severity results calculated during the present study (Figure 3.4). FHB severity showed a significant ($P \leq 0.001$) positive correlation with accumulation of DON ($r = 0.951$), D3G ($r = 0.887$), and 3-ADON (0.874) during disease progression in wheat spikes (Figure 4.3). DON accumulation was positively correlated with D3G ($r = 0.895$) and 3-ADON ($r = 0.905$) concentrations during FHB disease development ($P \leq 0.001$). A significant positive correlation ($r = 0.751$; $P \leq 0.001$) was also observed between D3G and 3-ADON concentration during FHB disease development in wheat spikes (Figure 4.3). Conversely, D3G to DON ratio showed a negative correlation ($r = -0.569$, $P \leq 0.01$) with FHB severity.

4.5 Discussion

Fusarium Head blight is a very serious wheat disease with detrimental effects on both yield and grain quality. Developing spikes are the primary targets for fungal infection, therefore an immature spike culture-based FHB screening technique (Sharma et al., 2018) is an efficient strategy to distinguish FHB resistant and susceptible genotypes. Both the methods PSS (Bai and Shaner, 1994) and visual rating scale (Stack and McMullen, 1995) distinguished the resistant (Sumai-3) and susceptible (AC Nanda) genotypes (Figure 3.4). Oliver et al. (2008) used Sumai-3 and "Russ" as resistant and susceptible controls respectively in greenhouse (three seasons) and field evaluations (two locations) to screen for FHB severity of wheat accessions. The PSS based FHB severity of Sumai-3 varied from 6.32 to 12.02 through three greenhouse trials after three weeks of inoculation; while the susceptible control Russ ranged from 28.40 to 79.04. In field nurseries, FHB severity of Sumai-3 varied from 0.64 to 13.16 across two locations; while Russ is rated from 25.04 to



	FHB severity	DON	D3G
DON	0.951***		
D3G	0.887***	0.895***	
3-ADON	0.874***	0.905***	0.751***

*** significant at $P \leq 0.001$.

Figure 4.3: Scatter plot (scatter graph) showing association of *Fusarium* head blight severity to accumulation of mycotoxins [deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), and 3-acetyldeoxynivalenol (3-ADON)] and ratio of D3G to DON, confirmed by Pearson's correlation coefficients (table) significant at $P \leq 0.01$ (**) and 0.001 (***).

75.64 at two locations. Jin et al. (2013) also used Sumai-3 as a resistant genotype in both greenhouse and field tests to examine the FHB severity of wheat accessions. In their study, also the PSS based FHB severity of Sumai-3 was rated 8.6 ± 3.6 in greenhouse, while in field test, it was rated 21.5 ± 18.0. The susceptible control "Duster" was rated 81.3 ± 18.7 and 85.9 ± 8.3, in greenhouse and field tests, respectively. However, in this study, PSS at 9 DAI after inoculation with fungal spores showed the most differences between the resistant (Sumai-3) and susceptible (AC Nanda) cultivars. The PSS for the resistant control (Sumai-3) is higher than reported in other studies (Oliver et al., 2008; Jin et al., 2013), but this could be due to the *in vitro* screening method using immature spike culture. To confirm the differences between the FHB susceptible and resistant genotypes, mycotoxin accumulation in the mature grain was analyzed using LC-MS/MS.

LC-MS/MS is a widely accepted method to analyze compounds present in very low concentrations in a biological sample. Therefore, LC-MS/MS based methods have been reported previously for determining concentrations of NIV, DON, D3G, 15-ADON and 3-ADON in FHB infected samples (Kokkonen and Jestoi, 2009; Yoshinari et al., 2012; Habler et al., 2017; Zuo et al., 2018). In this study all the mycotoxins were separated within 15 min of run time and the remaining 10 min contributed to column cleaning and preparation for the next injection. The R^2 values of >0.99 for all the compounds, lower LOD/LOQ values, >80% recovery percentage, and higher repeatability/intermediate precision confirmed the linearity, sensitivity, accuracy and precision of the optimized method. The compounds in wheat matrix using the optimized method showed similar SSE patterns suggesting no matrix effect on mycotoxins and confirming >80% recovery using the optimized method. The modified extraction method omitted the purification and drying steps without compromising mycotoxin separation in LC-MS/MS therefore, it can be characterized as a rapid, easy and economic method. The optimized method used only 0.6 g ground grain sample to analyze mycotoxin concentrations which is significantly lower compared to other reported methods (5.0-25.0 g) (Kokkonen and Jestoi, 2009; Yoshinari et al., 2012; Zuo et al., 2018) (Table 4.1). The methods reported by Yoshinari et al. (2012) and Zuo et al. (2018) had shorter runtime of 7 and 15 min whereas, methods reported by Kokkonen and Jestoi. (2009), and Habler et al. (2017) can determine 30 and 21 mycotoxins in a single LC-MS/MS run, respectively. However, these methods had higher LOD/LOQ

(0.5-241.7/1.3-500 ppb) but lower repeatability/intermediate precision (1.0-9.1/2.0-16.0% RSD) compared to the optimized method (0.30-0.75/0.5-2.5 ng mL-1 or ppb and 2.2-4.8/1.6-3.3% RSD) (Table 4.1). The optimized method (83.9-91.4% SSE) had significantly lower effect of wheat-matrix on mycotoxin determination than that of the method reported by Kokkonen and Jestoi (9.4-45.0% SSE) in 2009.

Using the optimized method, FHB -resistant and -susceptible genotypes showed distinct accumulation of DON, D3G and 3-ADON at all the intervals after fungal inoculation. FHB severity varied significantly between FHB -resistant and -susceptible genotypes at seven and nine DAI. FHB infection initiated around two DAI, but at five DAI, the FHB severity between the resistant and susceptible genotypes could be visibly distinguished but the difference was not statistically significant due to very high standard deviation in the susceptible genotypes. Therefore, FHB severity did not differ significantly at two and five DAI for FHB severity. In the present study, a linear association of FHB disease development to mycotoxin accumulation in wheat spikes, was observed in agreement with previous studies. A significant positive correlation between mycotoxin (DON and 3-ADON) concentrations and FHB severity have been reported in wheat (Tunali et al., 2006; Nobili et al., 2011; Palazzini et al., 2015; Sharma et al., 2018) and other small grain cereals (Wegulo, 2012) as free mycotoxin such as DON and 3-ADON are considered as virulence factors for Fusarium therefore, required for FHB incidence, progression and severity (Gunupuru et al., 2017). DON is the dominant mycotoxin produced by the *F. graminearum* isolate M7-07-1 followed by 3-ADON whereas D3G is the product of DON glucosylated by *UDP-Glucosyl Transferase*(s) (Sharma et al., 2018). The conversion of DON to D3G in plants allow less DON accumulation thus provide tolerance/resistance against FHB infection (Lemmens et al., 2005; Sharma et al., 2018). Therefore, a higher D3G:DON ratio was observed at seven and nine days after inoculation for FHB resistant genotypes (0.51-0.83 and 0.75-1.00 with an average of 0.63 and 0.85) compared to the susceptible genotypes (0.27-0.43 and 0.57-0.66 with an average of 0.33 and 0.61), respectively which led to a negative correlation between D3G:DON ratio and FHB severity agreeing with earlier reports. These results concurred with the conclusions of Ovando-Martínez et al. (2013) who reported a linear association between DON and D3G concentrations (Simsek et al., 2013) in FHB resistant hard red spring wheat genotypes

whereas this association was reversed in FHB susceptible genotypes. Besides DON, genetic, environmental and fungal chemotype also influence DON concentration and FHB incidence/severity/fungal biomass (Wegulo, 2012).

A simple extraction procedure coupled to LC-MS/MS method that used a solvent gradient was developed to separate and analyze selected *F. graminearum* induced mycotoxins in wheat spikes. The optimized method separated NIV, DON, D3G, 15-ADON, and 3-ADON in 25 min of total run time (separation completed in <15 min) with high coefficient of determination and recovery but low limits of detection and quantification thus had higher accuracy and precision compared to other LC-MS/MS based methods. The optimized method was used to demonstrate that FHB disease severity directly correlated to the accumulation of DON, 3-A DON and D3G, but inversely correlated to the D3G:DON (Figure 4.3). The LC-MS/MS method for mycotoxin accumulation can be utilized by plant pathologists, plant breeders and food scientists to identify these mycotoxin in a grain sample even when present in very low concentrations and to study the chemotype of *F. graminearum* isolate along with plant-microbe interaction analyses. The *in vitro* immature spike culture method can be used to identify FHB resistant germplasm and as a screening method in a breeding program to develop FHB resistant/tolerant crop varieties, respectively.

CHAPTER 5. *IN VITRO* WHEAT IMMATURE SPIKE CULTURE SCREENING IDENTIFIED FUSARIUM HEAD BLIGHT RESISTANCE CONFIRMED BY FIELD EVALUATION

Study 3

In this study, FHB resistant SCDV genotypes identified using immature spike culture were assessed in two Fusarium nurseries located at the University of Saskatchewan and University of Manitoba in Carman, MB, respectively.

CH conducted the experiments reported in the manuscript.

Huang, C., Gangola, M. P., Kutcher, H. R., Hucl, P., Ganeshan, S. and Chibbar, R. N. In vitro wheat immature spike culture screening identified fusarium head blight resistance confirmed by field evaluation. Submitted to Plant Disease.

5.1 Abstract

Fusarium head blight (FHB) is a devastating fungal disease of wheat (*Triticum aestivum* L.) resulting in reduced crop yield and nutritional quality due to the accumulation of mycotoxin in grain. The lack of genetic resources with stable FHB resistance combined with a reliable and rapid screening method to evaluate FHB resistance is a major limitation to develop FHB resistant wheat germplasm. The present study utilized an immature wheat spike culture method to screen wheat spike culture derived variants (SCDV) for FHB resistance. Mycotoxin concentrations determined by LC-MS/MS correlated significantly with FHB severity and disease progression during in vitro spike culture. In two Fusarium nursery field experiments, there was a significant effect of genotype, environment and their interaction on FHB resistance or severity among SCDV lines. The Fusarium incidence or severity during the field experiments was correlated with the disease severity of the selected SCDV lines during *in vitro* spike culture. The results corroborate the use of *in vitro* spike culture to identify FHB resistant lines that also showed FHB resistance in field conditions. The identified FHB resistant SCDV lines that can be used to develop FHB resistant wheat genotypes.

5.2 Introduction

Fusarium head blight (FHB) or scab is a major disease of wheat (*Triticum aestivum* L.) and is predominantly caused by *Fusarium graminearum* Schwabe [telomorph: *Gibberella zeae* (Schw.) Petch] (Rawat et al., 2016; Sharma et al., 2018). Although other Fusarium spp., including *F. avenaceum*, *F. culmorum*, and *F. poae*, also cause FHB, they are less frequently reported, and their incidence and disease severity is lower than *F. graminearum* (Desjardins, 2006). Fusarium spp. infect immature wheat spikes at the flowering stage when rainfall, high humidity, and warm night-time temperatures occur, and Fusarium infected crop residue is present. The disease restricts grain development and results in light shriveled kernels, which reduces yield (Gunupuru et al., 2017). Fusarium infection of wheat also produces the trichothecene group of mycotoxins including Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-ADON), 15-Acetyldeoxynivalenol (15-ADON), and Nivalenol (NIV) (Beres et al., 2018). The trichothecene group of mycotoxins inhibit eukaryotic protein synthesis, causing necrosis, chlorosis, and wilting of

spikes of infected plants (Lemmens et al., 2005). The mycotoxins, especially DON, also act as a virulence factor for the fungus by regulating its growth, development and responses to stimuli, thus facilitating disease spread within the spike (Gunupuru et al., 2017). The 3-ADON chemotype causes greater disease severity and DON accumulation compared to 15-ADON- and NIV- chemotypes (Puri and Zhong, 2010). Moreover, prevalence of 3-ADON chemotypes is increasing from east to west in Canada (Chakraborty and Newton, 2011; Goswami and Kistler, 2005; Ward et al., 2008); therefore, controlling the 3-ADON chemotype population is of high importance (Vujanovic et al., 2012). Additionally, contamination of food grains by these mycotoxin is a global challenge for plant and food scientists (Pierron et al., 2016) as their consumption even at very low concentrations (<2 ppm) can cause adverse gastrointestinal and immune-modulatory impacts on human and animal health (Gunupuru et al., 2017; Pierron et al., 2016; Sharma et al., 2018; Sobrova et al., 2010). To reduce the impact of DON or its derivatives, plants have *UDP-glucosyltransferase* (UGT) genes whose encoded enzyme can detoxify DON to Deoxynivalenol-3-Glucoside (D3G) with no (Pierron et al., 2016) or very low (Nagi et al., 2014) toxic effects on human or animal health; however, D3G can be reconverted to DON by intestinal microflora (Pierron et al., 2016).

FHB resistance in crops has been categorized in to three main groups, type I, II and III referring to resistance against initial infection or incidence, FHB progression, and mycotoxin accumulation, respectively (Sharma et al., 2018). In *in vitro* experiments, only FHB progression or type II resistance is evaluated as the initial infection starts from injecting inoculum in to a single spikelet of wheat whereas both disease incidences (type I) and severity (type II) are examined (Bai et al., 2018). Type II resistance is the most stable resistance therefore, it has been studied widely (Jin et al., 2013). To reduce the effect of FHB severity on wheat production and quality, several genetic and management practices have been suggested, among which development of FHB resistant or tolerant varieties is one of the most pragmatic strategy but it is also the most challenging (Beres et al., 2018; Jin et al., 2013). The poor agronomic performance of some resistant wheat genotypes such as Nobeokabouzu-Komugi, Sumai-3, Wangshuibai and Frontana, and moderate effectiveness under high disease severity of cultivars such as Canada Western Red Winter wheat cv. Emerson limit their adoption and utilization by producers (Beres et al., 2018).

Therefore, new stable genetic resources are required to develop FHB resistant or tolerant varieties in wheat breeding programs. The other major impediment to the development of FHB resistant wheat germplasm is lack of a direct rapid, precise and environment-independent method to identify FHB resistant genotypes. Several FHB screening methods including clip dipping, foliar spray, and pin point inoculation have been optimized and reported (Shin et al., 2014). With the clip dipping method, stems of three-day-old seedlings are wounded by pin point and inoculated with fungal suspension whereas, clip dipping and foliar spray methods screen 10-day-old seedlings infected with fungal suspension using dipping and spray methods, respectively. These are indirect methods to FHB screening and have not been validated with field experiments therefore, their use for germplasm screening may influence the selection process in breeding programs. Recently we have developed an immature wheat spike culture method to study grain development (Ganeshan et al., 2010) and to develop an ethyl methyl sulfonate treated spike culture derived variant (SCDV) wheat population (Ganeshan and Chibbar, 2017). The immature spike culture method was also modified to evaluate FHB severity or resistance (Sharma et al., 2018). The *in vitro* spike culture method is simple, rapid, environment-independent, and relatively less resource demanding (time and labor) compared to field-based screening (Sharma et al., 2018). To assess the utility of the immature spike culture method for FHB resistance, the grain from FHB–resistant SCDV lines was analyzed for mycotoxin concentrations using an LC-MS/MS method (Huang et al., 2018; unpublished data) and FHB resistance assessed in two field experiments in Fusarium inoculated field nurseries. The results indicated that grain mycotoxin concentrations were strongly correlated with FHB disease severity and progression that was observed in immature spike cultures. FHB resistant genotypes identified in immature spike cultures also showed FHB resistance in the field experiments.

5.3 Materials and Methods

5.3.1 Plant material

The present study utilized an M_3 population of ethyl methane sulfonate mutagenized spike culture derived variants (SCDVs) developed by Sharma et al. (2018). The 53 M_3 variants of the 134 SCDV lines were selected based on their consistent performance among replications (Sharma et al., 2018), and assessed along with Sumai-3

(FHB resistant) and AC Nanda (FHB susceptible) controls for FHB resistance using the optimized *in vitro* spike culture-based technique. In brief, M_3 seeds were grown in a greenhouse (University of Saskatchewan, Saskatoon, SK, Canada) during May to July 2015, and at least 20 immature spikes at the onset of heading were collected, grown on spike culture media, and point-inoculated with *F. graminearum* isolate M7-07-1 (moderately virulent, 3-ADON chemotype). The remaining spikes were grown to maturity and the M_4 seeds collected for further study. The FHB severity was recorded at 0, 5, 7, 9 and 11 DAI, whereas infected spikes of the selected genotypes (80, 93, 123, 136, AC Nanda, FHB202, FHB250, FHB252, FHB256 and Sumai-3) at 2, 5, 7, and 9 DAI (at least three spikes from each stage) were collected in liquid nitrogen and stored at -80ºC until further use to study the accumulation of mycotoxins and correlation with FHB severity. The leaves of these SCDV lines were also collected from a plant at the three-leaf stage and stored at -80ºC until DNA extraction.

Of the 53 SCDV lines, 13 (11 FHB resistant and 2 susceptible) were selected based on their contrasting FHB resistance. The M_4 seeds of the lines selected and Sumai-3 (FHB resistant control) and AC Nanda (FHB susceptible control) were utilized to evaluate their consistency for FHB resistance in in vitro spike culture- and field-based experiments during 2016. In the spike culture experiment, seeds of the M_4 SCDV lines, AC Nanda and Sumai-3 were grown in the greenhouse (University of Saskatchewan, Saskatoon, SK, Canada) under a 16/8 h day/night cycle with average daily temperature of 27.3/20.6ºC and relative humidity of 50.1/70.5% during May to July 2016. The immature spikes were collected, processed and FHB severity observed at 5, 7, 9, and 11 DAI (Sharma et al., 2018). The field experiments of the SCDV lines were performed in irrigated nurseries during May to August 2016 in an FHB field nursery (S2016) at the University of Saskatchewan, Saskatoon SK, Canada (52º 7’ N, 98º 0’ W, elevation 481.5 m) and FHB nursery (C2016) at Carman, MB Canada (49º 30’ N, 98º 0’ W, elevation 262 m). The average daily maximum/minimum temperature at S2016 and C2016 were 23.9/11.0ºC and 23.1/10.3ºC with total precipitation of 166.3 and 282.2 mm during the growing period, respectively. At C2016, the experiment was performed with two replications in a randomized complete block design as described (Ruan et al., 2012). At S2016, the experiment was performed in two replications in a completely randomized design in which SCDV lines were seeded in

hills. Each hill had 30 seeds of one SCDV line and hills were spaced at approximately 45 cm. The plants were inoculated at 50% anthesis by spreading corn spawn (~4 g per hill) infected with 3-ADON and 15-ADON chemotpyes. FHB incidence and severity were recorded to calculate a Visual Rating Index (VRI) as, VRI = (% incidence × % severity)/100, as described (Stack and McMullen, 1995).

5.3.2 Extraction and quantification of mycotoxins using LC-MS/MS

The FHB infected infected wheat spikes (three replications) were cut into two halves from the point of inoculation and the lower halves were used for mycotoxin extraction whereas the upper halves were stored at -80ºC for future genetic studies. Spikes were pooled, ground and homogenized using a mortar and pestle. The sample (0.6 g from three replications) was mixed with 4 mL of extraction buffer (acetonitrile:water; 84:16, v/v) in a 15 mL disposable tube. The mycotoxins extracted were analyzed with a 4000 QTRAP LC-MS/MS system (AB SCIEX, Framingham, MA, USA) that had a high-performance liquid chromatography equipment with an electrospray ionization (ESI) and a hybrid triple quadrupole ion trap tandem mass spectrometer (Huang et al., unpublished data). The analysis of chromatograms was performed using Analyst 1.6.2 software (AB SCIEX, Framingham, MA, USA).

The standards used for DON, 3-ADON, D3G, 15-ADON, and NIV were purchased from Romer Labs (Washington, MO, USA), whereas LC-MS grade water, LC-MS grade methanol, acetonitrile, acetic acid, and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The data for FHB severity and mycotoxin accumulation during *in vitro* spike culture for 123, 136, AC Nanda, FHB202, FHB256 and Sumai-3 were obtained from the unpublished data (Huang et al. unpublished data).

5.3.3 Validation of single nucleotide polymorphic marker in the selected SCDV lines

The leaves of the SCDV lines selected (80, 93, 123, 136, AC Nanda, FHB202, FHB250, FHB252, FHB256 and Sumai-3) were utilized to extract genomic DNA following the protocol of the Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). The

genomic DNA was used to validate the single nucleotide polymorphic marker on the *TaUGT-3B* gene using High Resolution Melt (HRM) Curve analysis (Sharma et al., 2018).

5.3.4 Statistical analysis

Multiple comparisons using Tukey's test were performed to compare disease severity at different days after inoculation, between resistant and susceptible genotypes, and during the two field experiments. A General Linear Model was used for the analysis of variance in order to assess the effect of genotype, environment, and their interaction on FHB severity. Correlation analysis was performed to analyze the association among the various traits studied whereas, multivariate analysis was performed to group the genotypes based on the parameters studied. The statistical analyses were performed using Proc Mixed of software SAS 9.4 (SAS Institute, Cary, North Carolina, USA).

5.4 Results

5.4.1 Variation for FHB resistance in the SCDV population

The 53 SCDV lines (M_3 stage) evaluated using the *in vitro* spike culture technique varied for FHB resistance ($P < 0.001$) from 24.3 to 75.5%, 29.3 to 80.1%, 43.7 to 84.1%, and 56.7 to 100% with averages of 43.8%, 60.5%, 71.7% and 89.4% at 5, 7, 9 and 11 days after inoculation (DAI), respectively (Figure 5.1). Replication effect was not significant at any stage after inoculation. The FHB severity at the M_2 stage (Sharma et al., 2018) was weakly correlated with that of the M_3 stage at 7 DAI ($r = 0.326$, $P < 0.05$) and 9 DAI ($r = 0.322$, $P < 0.05$). The FHB resistant and susceptible SCDV lines at the M_3 stage differed from each other at 7 and 9 DAI ($P \leq 0.001$) only. FHB severity varied between resistant and susceptible lines identified by genetic and phenotypic study at the M_2 stage ($P < 0.001$). High resolution melt (HRM) curve analysis on the selected SCDV variants using *TaUGT-3B* primer in the nested real-time PCR, showed that AC Nanda and other 4 susceptible genotypes (80, 93, 123, 136), with nucleotide G at 1558 bp; while the second group include Sumai-3 and resistant EMS mutants (FHB202, FHB252, FHB250, FHB256), had the nucleotide T at the same position (data not shown).

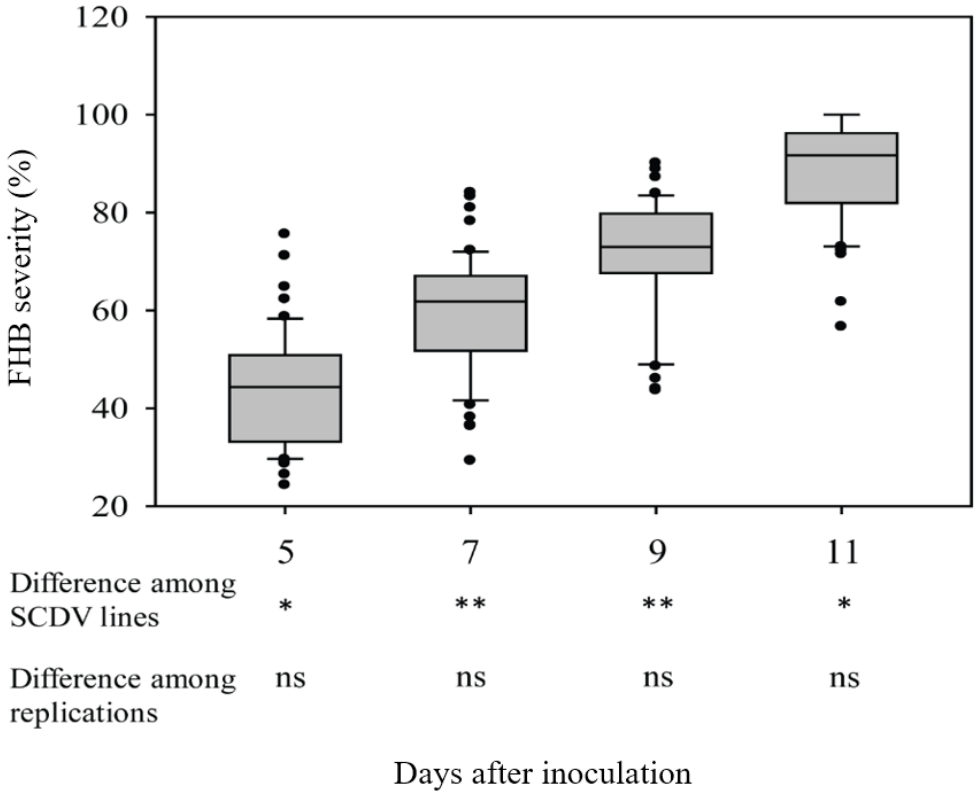


Figure 5.1: Box-plot analysis of 53 spike culture derived variants (SCDV) showing variation for Fusarium head blight (FHB) severity at 5, 7, 9, and 11 days after inoculation. Replications did not differ from each other for FHB severity at any stage; therefore, this was denoted by ns = not significant. The upper and lower error bars indicate the non-outlier maximum and minimum range of the data set. The box represents the interquartile range (IQR), whereas the middle line is the median value of the data set. The dark circles represent the outliers, calculated as the data points out of 1.5 times the IQR.

5.4.2 FHB resistant SCDV lines had reduced mycotoxin accumulation compared with susceptible lines

The FHB severity ranged from 22.4 to 26.4%, 32.5 to 43.0%, 40.0 to 56.1%, and 73.0 to 77.5% in FHB resistant SCDV lines at 5, 7, 9 and 11 DAI, which was lower than the 43.0 to 56.9%, 61.0 to 69.4%, 74.6 to 80.1%, and 95.6 to 100.0% FHB severity of the FHB susceptible SCDV lines ($P \leq 0.001$), respectively (Figure 5.2). DON was the predominant mycotoxin produced by *F. graminearum* isolate M7-07-1, followed by 3-ADON, whereas, 15-ADON/nivalenol were either absent or not detected and D3G was a glucosylated product of DON. The FHB susceptible SCDV lines accumulated higher concentrations of DON (7.1-12.3, 13.0-17.8, 15.0-20.1, and 9.6-12.1 mg kg^{-1}), D3G (3.5-6.5, 3.7-7.6, 8.6-14.3, and 8.5-9.1 mg kg^{-1}) and 3-ADON (5.8-6.3, 4.3-7.0, 5.2-8.3, and 3.2-5.7 mg kg^{-1}). On the other hand, the FHB resistant SCDV lines had lower DON concentrations (3.1-4.8, 3.0-5.9, 2.4-6.5, and 3.8-5.2 mg kg-1), D3G (1.7-5.0, 2.4-3.2, 2.4-4.9, and 2.6-5.8 mg kg^{-1}) and 3-ADON (1.1-2.6, 0.9-2.9, 1.3-2.7, and 1.5-2.6 mg kg^{-1}) at various intervals (5, 7, 9 and 11 DAI) of FHB disease development, respectively (Figure 5.2). DON and 3-ADON concentrations varied ($P \leq 0.001$) between resistant and susceptible SCDV lines at 5, 7, 9 and 11 DAI, whereas, D3G concentrations differed ($P \leq 0.001$) only at 7, 9, and 11 DAI.

5.4.3 Genotype by environment interaction affected FHB severity

In the spike culture experiment, FHB severity increased from 20.7-62.3% at 5 DAI to 26.2-78.2% at 7 DAI, 44.4-83.0% at 9 DAI, and 72.2-96.3% at 11 DAI; averages of 40.3, 55.9, 68.3 and 88.0%, respectively (Figure 5.3A). In two field experiments, S2016 had lower FHB severity (4.5-32.5%) and VRI (3.9-21.0) compared to that of C2016 (10-50% FHB severity and 5.0-43.8% VRI) ($P < 0.05$); whereas, FHB incidence did not vary between S2016 (30.5-100%) and C2016 (50-80%) (Figure 5.3B). Analysis of variance (ANOVA) using the general linear model (Mean sum of squares=MSS) indicated a significant ($P < 0.001$) effect of genotypes (MSS=441.2), environment (MSS=8374.6), and their interaction (MSS=331.2) on FHB severity, whereas there was no difference between the two biological replications (MSS=4.1, $P > 0.05$).

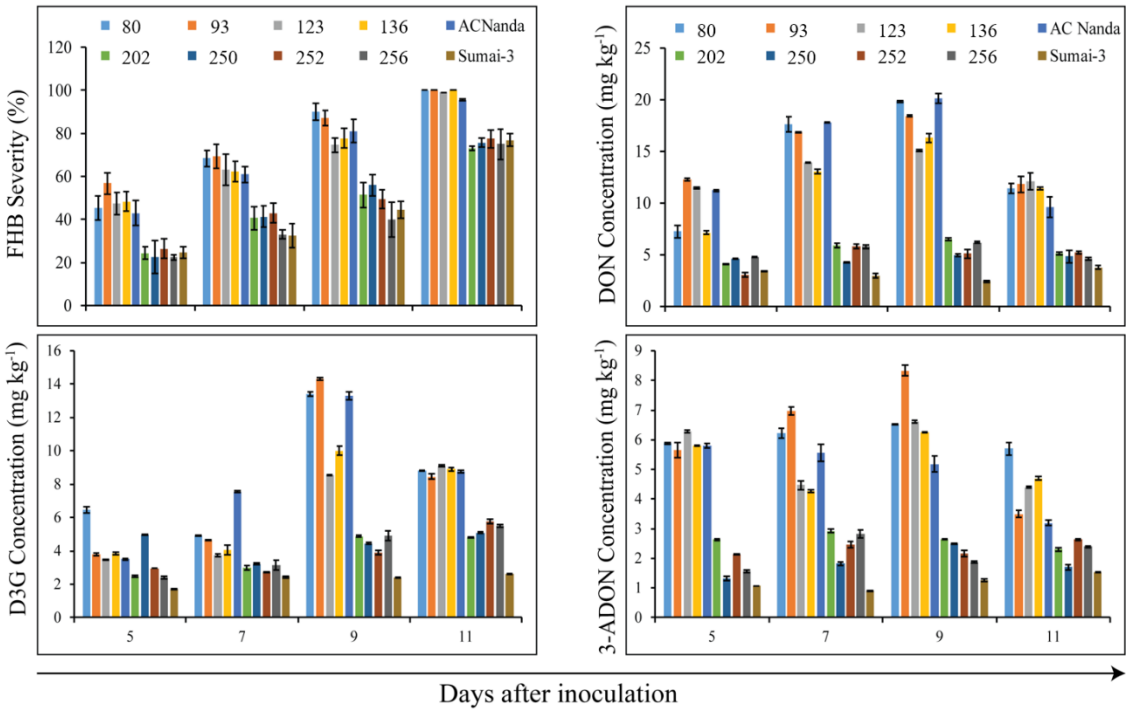


Figure 5.2: Bar-graphs showing FHB severity (%), and concentrations (mg/kg) of deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G) and 3-acetyldeoxynivalenol (3-ADON) in FHB resistant and susceptible SCDV lines at 5, 7, 9, and 11 days after inoculation.

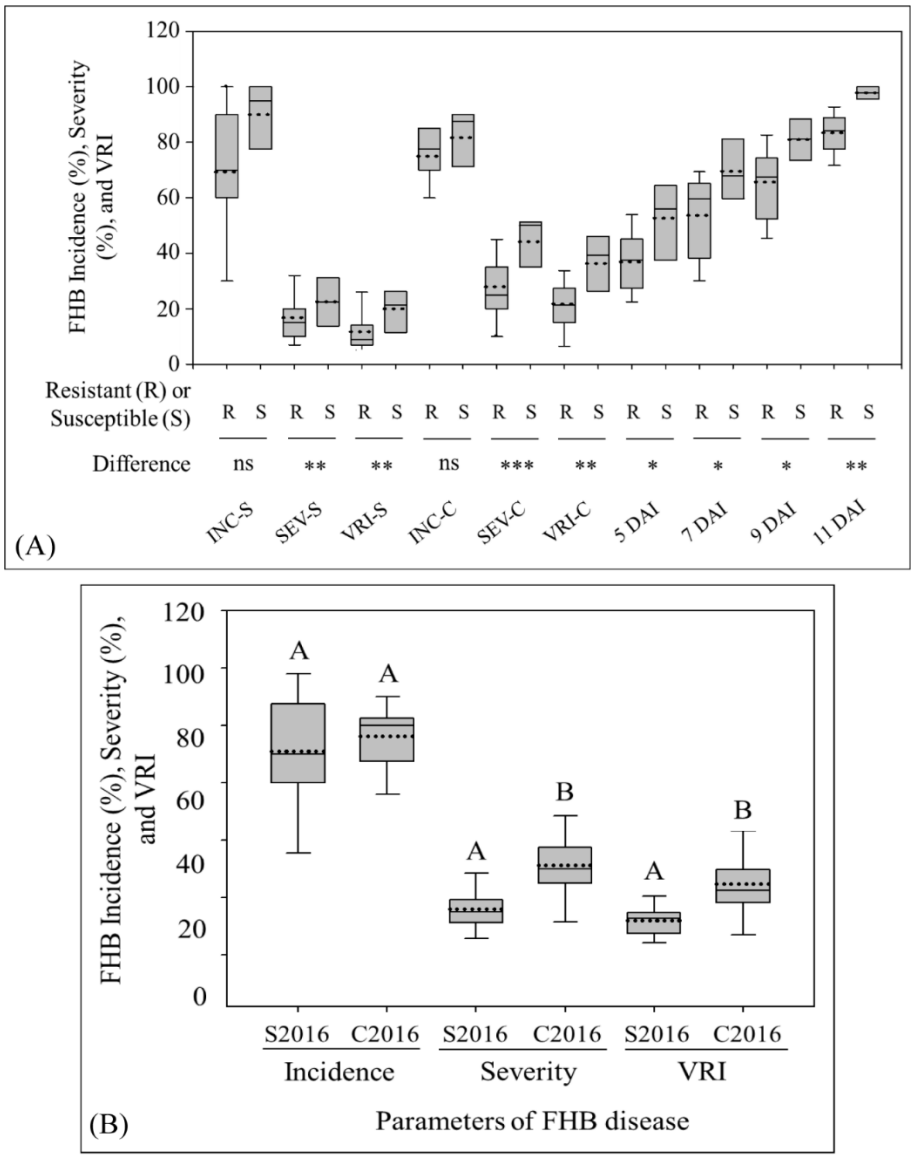


Figure 5.3: Box-plot analysis comparing, (A): resistant (R) and susceptible (S) spike culture derived wheat variants (SCDV) for incidence (INC), severity (SEV), and visual rating index (VRI) in field experiments at Saskatoon (S) and Carman (C), whereas only severity was compared in the in vitro spike culture experiment at 5, 7, 9 and 11 days after inoculation (DAI). *, ** and *** indicate significant differences at $P \leq 0.05$, 0.01, and 0.001, respectively; ns represents non-significant; and (B): SCDV lines grown in field experiments at Saskatoon (S2016) and Carman (C2016); different letters over the boxes indicate significant difference at $P \leq 0.001$. The upper and lower error bars indicate the non-outlier maximum and minimum range of the data set. The box represents the interquartile range (IQR), whereas the middle continous and dotted lines are the median and average values of the data set, respectively.

5.4.4 Correlation between spike culture and field experiments for FHB severity of SCDV lines

FHB severity at 7 and 11 DAI during spike culture was positively correlated with severity ($r = 0.501$ and 0.602, $P \leq 0.01$ and 0.001) and VRI ($r = 0.562$ and 0.544, $P \leq 0.01$ and 0.01) in C2016; whereas, FHB severity at 9 DAI during spike culture was positively correlated with disease incidence ($r = 0.398$ and 0.443; $P \leq 0.05$) for both the field experiments (C 2016 and S2016), but with severity ($r = 0.583$, $P \leq 0.01$) and VRI ($r = 0.604$, $P \leq 0.001$) in C2016 only (Table 5.1). However, FHB severity was very low at 5 DAI during spike culture and hence there was no correlation with the parameters of the field experiments. The FHB incidence in S2016 was positively correlated with incidence ($r = 0.448$), severity ($r = 0.430$) and VRI ($r = 0.404$) in C2016 ($P \leq 0.05$). The VRI in S2016 was also positively correlated ($r = 0.367$, $P \leq 0.05$) with FHB severity in C2016. The disease severity values in S2016 were lower than that of C2016 and spike culture was not correlated with most of the parameters of S2016.

5.5 Discussion

A population of spike culture derived variants (SCDV) developed in a previous study (Sharma et al., 2018) for FHB resistance was utilized in this study. The 53 SCDV lines selected based on their FHB resistance at the M_2 stage carried their resistance over to the M_3 stage, which was confirmed by the significant variation in their FHB resistance and its correlation with that of the M_2 stage. Lack of differences among replications at all stages after fungal inoculation affirmed the repeatability of the spike culture technique in evaluating FHB resistance *in vitro*. The correlation between the M_2 and M_3 stages for FHB resistance or severity indicated a moderately to highly heritable trait that coincided with the heritability range of 0.5-0.9 for distinct FHB related parameters in wheat (Burt et al., 2015; Miedaner et al., 2017). The source of variation for FHB resistance among SCDV lines was attributed to single nucleotide polymorphisms in two *UDP-glucosyltransferase* (*UGT*) genes, *TaUGT-2B* and *TaUGT-3B* (Sharma et al., 2018), which encode the *UDP-glucosyltransferase* enzyme catalyzing the glycosylation of DON in to D3G that is non-toxic to plants (Rawat et al., 2016).

Table 5.1: Correlation analysis among *Fusarium* head blight (FHB) severity assessment factors and times (days after inoculation, DAI) using the *in vitro* spike culture technique to assess FHB incidence, severity, and visual rating index during field experiments at Saskatoon and Carman.

	INC-S2016	SEV-S2016	VRI-S2016	INC-C2016	SEV-C2016	VRI-C2016	5 DAI	7 DAI	9 DAI
SEV-S2016	0.108ns								
VRI-S2016	0.507**	0.875***							
INC-C2016	0.448*	0.063ns	0.163ns						
SEV-C2016	0.430*	0.116ns	0.367*	0.488**					
VRI-C2016	0.404*	0.005ns	0.238ns	0.665***	0.861***				
5 DAI	0.261ns	0.142ns	0.299ns	0.328ns	0.375ns	0.368ns			
7 DAI	0.246ns	0.003ns	0.162ns	0.370ns	0.501**	0.562**	0.709***		
9 DAI	0.443*	0.070ns	0.191ns	0.398*	0.583**	0.604***	0.608***	0.840***	
11 DAI	0.350ns	0.250ns	0.386ns	0.306ns	0.602***	0.544**	0.428*	0.558**	0.522**

INC-S2016/INC-C2016, SEV-S2016/SEV-C2016, and VRI-S2016/VRI/C2016 represent incidence, severity, and visual rating index during field experiments at Saskatoon, SK and Carman, MB in 2016, whereas, 5, 7, 9 and 11 indicate DAI during *in vitro* spike culture assessment. *, ** and *** indicate significant differences at $p \leq 0.05$, 0.01, and 0.001, respectively; ns - non-significant difference.

The selection of individuals from the progeny of a cross determines the success of a typical breeding program. Therefore, FHB evaluation is a critical factor that needs to be performed accurately and precisely. At present, field-based inoculation (to the soil surface and into single florets) and other approaches including the detached leaf assay, the clip dipping method, the foliar spray method and pin-point inoculation have been reportedly used to evaluate FHB severity or resistance (Kumar et al., 2011; Shin et al., 2014; Yang et al., 1999). Field based FHB screening is reliable but is affected by environmental conditions, and is time and labor intensive, whereas other approaches are faster and utilize leaves instead of spikes. FHB mainly affects spikes at the flowering stage; therefore, evaluating FHB resistance in detached leaves is an indirect approach and may affect the selection process. Consequently, an FHB screening method is required that combines the easy and fast approach of indirect methods with the reliability of field-based screening methods. An *in vitro* spike culture method optimized by Ganeshan and Chibbar (2017), and Sharma et al. (2018) to assess FHB severity was evaluated in the present study for its accuracy and precision by correlating FHB severity values with mycotoxins accumulation and field-based screening results.

To understand the correlation between FHB severity and mycotoxin accumulation, FHB resistant (FHB244, FHB250, FHB252, FHB256 and Sumai-3) and susceptible (80, 93, 123, 136 and AC Nanda) SCDV lines (M_3 stage) were compared at various days after FHB inoculation. An LC-MS/MS based approach was optimized eluting all the mycotoxin in a total runtime of 25 min, of which 15 min was utilized to elute the mycotoxins and the remaining 10 min for column cleaning and preparation for the next injection. Each sample in an LC-MS/MS was run in two parts: (i) negative mode was up to 12 min as all compounds (NIV, DON and D3G) were of negative polarity, and (ii) 12 to 25 min in both positive and negative modes as 15-ADON (positive polarity) and 3-ADON (negative polarity) were eluted at the same retention time (13.4 min) but in separate modes; therefore, running both modes was necessary to analyze these two compounds (Huang et al., unpublished data). The increased FHB development or severity corresponded with increased mycotoxin accumulation in FHB susceptible SCDV lines compared to their resistant counterparts. Therefore, a positive correlation was identified between FHB severity and mycotoxins accumulation at most time intervals after fungal inoculation.

Consequently, multivariate analysis using FHB severity, and concentrations of DON, D3G and 3-ADON distinctly grouped FHB resistant and susceptible SCDV lines (Figure 5.4). The SNP marker at 1,558 bp from the translation initiation site on *TaUGT-3B* associated with FHB resistance also distinctly categorized FHB resistant and susceptible lines (Figure 5.4). The mycotoxin produced by some Fusarium spp. increase its infectivity, pathogenicity, and virulence thus influencing infection, disease development and severity (Gunupuru et al., 2017). The results were in agreement with the correlation studies reported by Bjørnstad et al. (2017), Gorczyca et al. (2018), Legzdina and Buerstmayr (2004), and Touati-Hattab et al. (2016).

The resistant and susceptible SCDV lines differed during *in vitro* spike culture and two field experiments (Figure 5.3A). Disease severity was maximized in the *in vitro* spike culture experiment due to favorable growing conditions for the pathogen. The higher FHB severity in C2016 compared to S2016 can be explained by the difference in precipitation that occurred during the growing season at each location. At C2016, a total 282.2 mm of precipitation was received with an average of 3.1 mm/day, whereas at S2016 precipitation was 166.3 mm with a mean of 1.8 mm/day. High humidity or intensive precipitation events support the survival of the primary inoculum during the vegetative stage, and augment infection during the anthesis stage of plant development, thus facilitating FHB development (Audenaert et al., 2013; Beres et al., 2018; Shah et al., 2018). Consequently, these results agree with the suggestions made by Beres et al. (2018), Miedaner et al. (2001), and Scala et al. (2016).

The FHB severity determinations using the *in vitro* spike culture technique were positively correlated with the field assessments, especially for the values obtained in C2016. This correlation is in agreement with the conslusion of Jin et al. (2013) who also reported a strong positive correlation ($r = 0.73$, $P < 0.001$) between FHB severity observed during green house and field experiments of 363 U.S. winter wheat accessions. These correlations validate the utilization of the *in vitro* spike culture technique to evaluate FHB resistance or severity (Sharma et al., 2018).

In summary, the present study validated the in vitro spike culture technique for the evaluation of FHB severity or resistance. FHB resistant SCDV lines had reduced DON, 3-ADON and D3G accumulation compared to that of FHB susceptible SCDV lines.

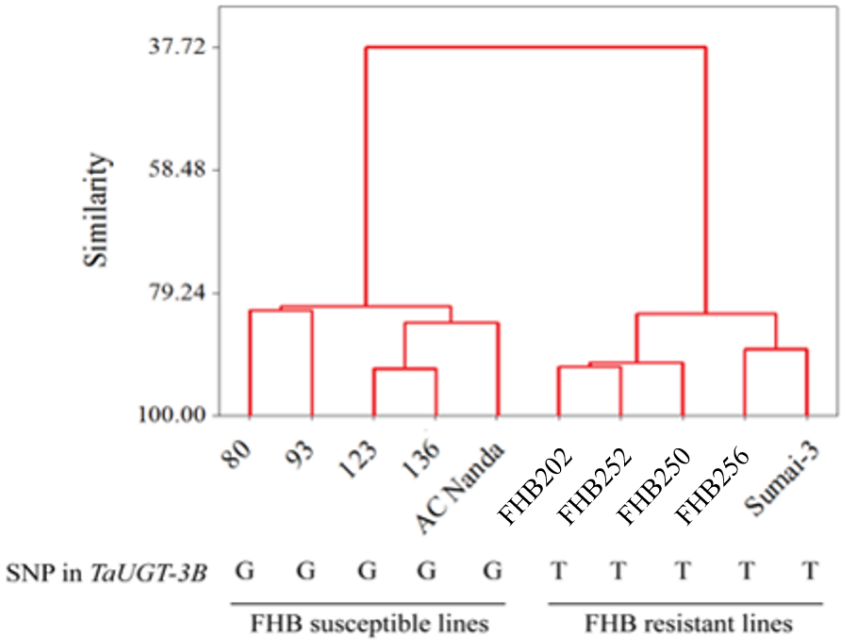


Figure 5.4: Multivariate analysis categorizing FHB -resistant (FHB202, FHB252, FHB250, FHB256, and Sumai-3) and -susceptible (80, 93, 123, 136, and AC Nanda) SCDV lines into two distinct groups based on FHB severity and mycotoxin concentration during FHB infection. The single nucleotide polymorphic marker (SNP) at 1,558 bp from the translation initiation site on *TaUGT-3B* also indicated a difference between FHB -resistant and -susceptible genotypes.

Consequently, a positive correlation was observed between FHB severity and mycotoxin accumulation during FHB disease development; therefore, multivariate analysis based on these parameters categorized FHB resistant and susceptible SCDV lines into two distinct groups. The SCDV lines were also evaluated in field experiments for FHB resistance; there was a correlation with the *in vitro* spike culture results. The use of the *in vitro* spike culture technique to separate FHB resistant and susceptible SCDV lines validated our hypothesis that this technique can be utilized to accelerate breeding programs aimed at developing FHB resistant wheat varieties.

CHAPTER 6. IDENTIFICATION OF SPIKE CULTURE DERIVED WHEAT (*TRITICUM AESTIVUM* L.) VARIANTS RESISTANT TO MULTIPLE ISOLATES OF *FUSARIUM GRAMINEARUM*

Study 4

The main objective of this study was to assess the immature spike culture-based screening for FHB using five *F. graminearum* isolates producing different mycotoxins and correlate it with mycotoxin concentrations in the spikes.

CH conducted the experiments reported in the manuscript.

Huang, C., Gangola, M. P., Hucl, P., Kutcher H.R. and Chibbar, R. N. (2018). Identification of spike culture derived wheat (*Triticum aestivum* L.) variants resistant to multiple chemotypes (isolates) of *Fusarium graminearum*. (to be submitted).

6.1 Abstract

Fusarium graminearum, the predominant species causing Fusarium head blight (FHB) in wheat (*Triticum aestivum* L.), can be sub-grouped in to three chemotypes producing, (i) 3-Acetyldeoxynivalenol (3-ADON), (ii) 15-Acetyldeoxynivalenol (15-ADON), and (iii) Nivalenol (NIV) mycotoxins, that differ in aggressiveness and response to fungicides. Moreover, prevalence of these chemotypes changes over time. Therefore, to develop wheat variety (ies) having resistance to multiple chemotypes, total of five chemotypes (mycotoxin produced) of *F. graminearum*, Carman-NIV (NIV), Carman-705-2 (3-ADON)), M9-07-1 (3-ADON), M1-07-2 (15-ADON), and China-Fg809 (15-ADON), were identified using an LC-MS/MS based method. These chemotypes were used to evaluate FHB severity in 55 spike culture derived variants following an optimized *in vitro* spike culture technique. The SCDV genotypes FHB213.4, FHB244.1, FHB245.6, FHB250.2, and FHB252.3 showed resistance to multiple chemotypes. The five SCDV can be utilized in breeding programs to develop wheat varieties with improved FHB resistance.

6.2 Introduction

Fusarium head blight (FHB) is one of the major fungal diseases of wheat (*Triticum aestivum* L.), caused by various Fusarium species among which *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein. Petch) is the most predominant in the North American region (Sharma et al., 2018). High humidity and warm temperatures favor the development of FHB in wheat during the flowering and early grain-fill stages in the presence of abundant natural inoculum (Bai and Shaner, 1994; McMullen et al., 1997; Zhou et al., 2002; Simsek et al., 2012) restricting grain development, producing light shrivelled grains and reducing grain yield (Gunupuru et al., 2017). FHB epidemics in 1993 (Manitoba in Canada whereas Minnesota, North Dakota and South Dakota in USA), 1996 (Ontario, Canada), and 1991-1997 (USA) in North American region caused >US$ 1 billion, >CA$ 100 million, and >US$ 1.3 billion losses to wheat production and export (Shah et al., 2018). FHB also diminishes crop value by accumulating the trichothecene group of mycotoxins (Figure 2.2) in infected grains, including Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-ADON), 15-Acetyldeoxynivalenol (15-ADON), and Nivalenol (NIV) (Beres et al., 2018). DON in plants can be glucosylated in to Deoxynivalenol-3-

glucoside (D3G) in an *UDP-Glucosyltransferase* (EC 2.4.1.x) catalyzed reaction. These mycotoxins facilitate fungal growth, development, and response to stimuli thus considered as fungal virulent factors (Gunupuru et al., 2017). The 3-ADON chemotypes, cause almost two times higher mycotoxin accumulation thus higher FHB severity compared to 15-ADON and NIV chemotypes (Puri and Zhong, 2010), are becoming more prevalent in North America, Asia and Europe (Goswami and Kistler, 2005; Ward et al., 2008; Chakraborty and Newton, 2011). These mycotoxins, especially DON are highly stable compounds, and do not degrade at elevated temperatures, or during the milling, storage or food processing stages (Simsek et al., 2012). The consumption of food or feed contaminated with DON adversely affect gastrointestinal and immune-modulatory systems in humans and animals (Gunupuru et al., 2017; Sobrova et al., 2010; Pierron et al., 2016). Consequently, a maximum regulatory concentration of 200-2000 ppb is permitted in cereals in approximately 37 countries (Nakagawa et al., 2017) including Asian countries (500-2000 ppb; Anukul et al., 2013), European countries (200-1750 ppb; Siegel and Babuscio, 2011), and Canada (2000 ppb; Yuan et al., 2017). Besides DON, *F. graminearum* also produces and accumulates other mycotoxin including 3-ADON, 15-ADON, and NIV, depending on chemotype of the isolate. However, no single *F. graminearum* isolate has been reported to simultaneously produce DON and NIV (Amarasinghe et al., 2015). Consequently, regulations across the globe are also limiting the NIV concentrations from 20–60 µg/kg in France to 584–1780 µg/kg in China (Kongkapan et al., 2016). Prevalence of these chemotypes changes over the years as influenced by environmental factors, e.g. a striking predominance of 3-ADON chemotypes replacing 15-ADON chemotypes with in six years in Canada (Kelly et al., 2015; Li et al., 2017). Most of the studies to the date have mainly concentrated on resistance to DON, the most widely present chemotype of the present time. However, the impact of DON may decfease if there is change in chemotypes prevalence and co-occurrence of multiple isolates producing distinct chemotypes. The present study identified spike culture derived variants exhibiting improved resistance to multiple chemotypes that can be utilized in breeding programs to potentially develop wheat varieties with a broad spectrum resistance to FHB.

6.3 Materials and methods

6.3.1 Identification of different chemotypes of *F. graminearum*

A total of five *F. graminearum* strains (M9-07-1, M1-07-2, Carman-NIV, China-Fg809, and Carman-705-2) were procured from the former Cereal Research Centre (Agriculture and Agri-Food Canada, Winnipeg, MB, Canada). To determine their chemotype, AC Nanda (a soft white spring wheat variety) seeds were grown in agricultural greenhouse (University of Saskatchewan, Saskatoon, SK, Canada). The spikes were collected at the onset of heading stage, point inoculated with five strains, and collected at 2, 5, 7, 9, and 11 days after inoculation (DAI) as described (Sharma et al., 2018). The disease severity data was also recorded at these DAI to calculate its correlation to mycotoxin accumulation. The collected spikes were utilized to extract the mycotoxins, which were identified and determined by an optimized LC-MS/MS based approach (Huang et al., unpublished data).

6.3.2 Identification of SCDV lines having resistance against multiple isolates

The Fifty-five spike culture derived variants (SCDV) were selected based on their FHB resistance from a population of 134 SCDV lines developed previously (Chapter 3). The selected M_3 SCDV lines were grown in a greenhouse during May-July 2016 with 16/8 h of day/night cycle with average daily temperature of 27.3/20.6°C and relative humidity of 50.1/70.5%. The immature spikes were collected at the onset of the heading stage, maintained in spike culture media *in vitro,* and point-inoculated with the five strains of *F. graminearum* as described (Sharma et al., 2018). The data for FHB severity was observed at 5, 7, 9, and 11 days after inoculation (DAI), and calculated as: FHB severity (%) = [(number of infected spikelets / Total number of spikelets) × 100].

6.3.3 Statistical Analysis

The multiple comparisons using Tukey's method, multivariate analysis, and correlation analysis were performed using Proc Mixed of software SAS 9.4 (SAS Inc., Cary, NC, USA).

6.4 Results

6.4.1 The five isolates of *F. graminearum* showed distinct chemotypes

The five isolates of *F. graminearum* grouped into two categories: (1) nivalenol producing chemotype that included only Carman-NIV, and (2) DON and derivatives producing chemotypes including M9-07-1, M1-07-2, China-Fg809, and Carman-705-2. Wheat ispikes infected with Carman-NIV accumulated mainly nivalenol and the concentration increased from 0.2 mg/kg at 2 DAI to 2.4 mg/kg at 5 DAI, 3.9 mg/kg at 7 DAI, 12.6 mg/kg at 9 DAI and decreased thereafter to 3.7 mg/kg at 11 DAI. The other three mycotoxins (0.0-66.9 µg/kg) and D3G (6.4-50.2 µg/kg) were present in very small concentrations compared to nivalenol (Figure 6.1). The isolate M1-07-2 infected spikes accumulated 1.3, 6.1, 17.0, 24.4 and 27.8 mg/kg of DON; 0.1, 0.2, 0.3, 0.5 and 1.0 mg/kg of 3-ADON, and 0.7, 1.9, 1.9, 1.9 and 2.7 mg/kg of 15-ADON at 2, 5, 7, 9 and 11 DAI, respectively. Similarly, China-Fg809 infected spikes, the DON concentration was 0.8, 3.2, 9.9, 12.5 and 7.0 mg/kg; 3-ADON/15-ADON concentrations were 0.1/0.4, 0.2/2.1, 0.4/1.8, 0.3/0.7 and 0.3/1.7 mg/kg in at 2, 5, 7, 9 and 11 DAI, respectively. Carman-705-2 infected spikes accumulated 2.1, 8.7, 13.8, 20.3 and 13.2 mg/kg of DON; 1.1, 1.1, 2.4, 3.3 and 3.3 mg/kg of 3-ADON at 2, 5, 7, 9 and 11 DAI. The M9-07-1 infected spikes had 1.5, 10.3, 17.1, 18.3 and 17.5 mg/kg of DON; 1.2, 3.3, 2.8, 2.7 and 7.1 mg/kg of 3-ADON at 2, 5, 7, 9 and 11 DAI, respectively. D3G was either absent or present at trace concentration in Carman-NIV infected spikes. D3G concentration varied between 0.3-0.9, 0.9-7.4, 6.0-9.6, 8.8-15.9, and 2.6-18.4 mg/kg at 2, 5, 7, 9, and 11 DAI in M1-07-2, ChinaFg813, Carman-705-2 and M9-07-1. DON was the predominant mycotoxin in *F. graminearum* isolates other than Carman-NIV whereas, nivalenol (0.0-293.5 µg/kg) was present in very low concentrations compared to DON (Figure 6.1). Among DON producing isolates, Carman-705-2 and M9-07-1 were mainly 3-ADON producing strains whereas ChinaFg809 and M1-07-2 accumulated 15-ADON predominantly. Thus four isolates grouped together and were distinctly separate from Carman-NIV in the multivariate analysis based on mycotoxin accumulation at various days after fungal inoculation (Figure 6.2).

6.4.2 FHB disease development showed a significant correlation with mycotoxin accumulation

The correlation study was based on disease severity and mycotoxin accumulation

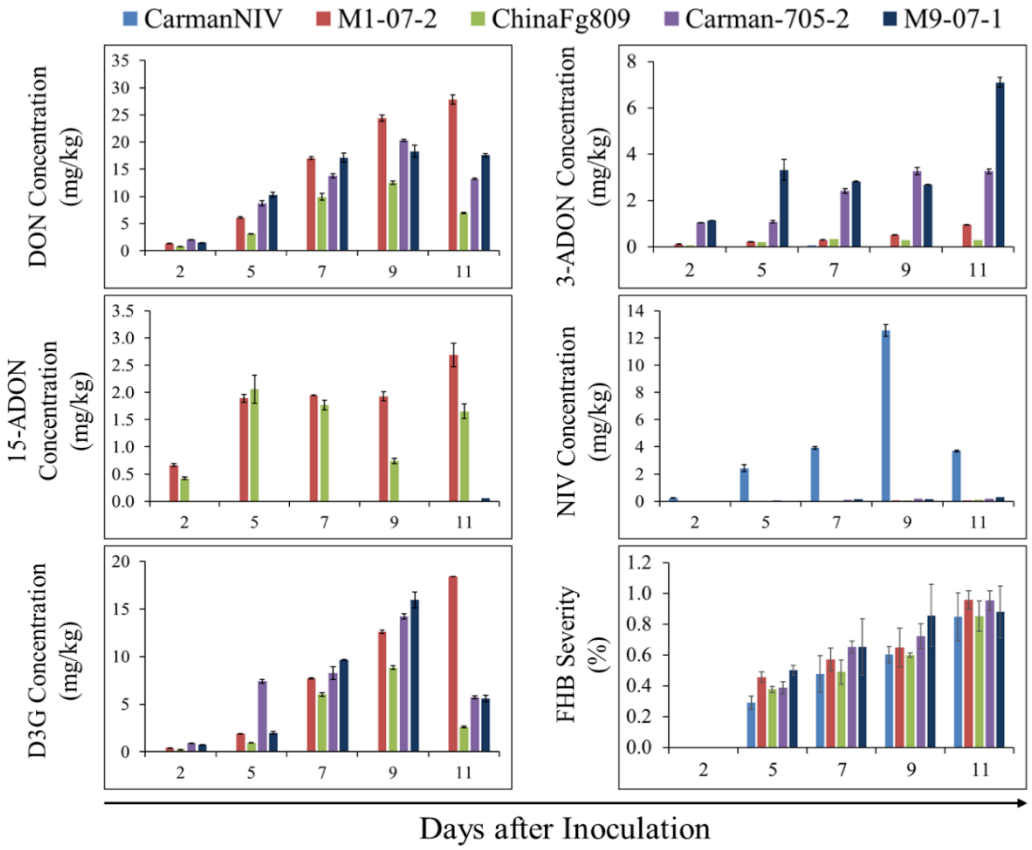


Figure 6.1: Accumulation of Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-ADON), 15-Acetyldeoxynivalenol (15-ADON), Nivalenol (NIV), and Deoxynivalenol-3-Glucoside (D3G) together with *Fusarium* Head Blight severity at 2, 5, 7, 9, and 11 days after inoculation with *F. graminearum* isolates (M9-07-1, M1-07-2, Carman-NIV, China-Fg809, and Carman-705-2).

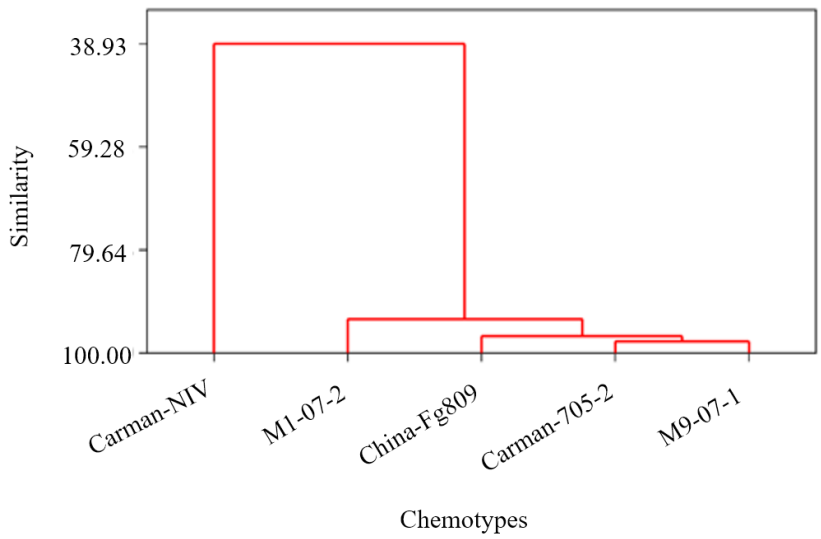


Figure 6.2: Multivariate analysis showing similarity (%) among distinct isolates of *Fusarium graminearum* – Carman-NIV produced Nivalenol, M1-07-2/China-Fg809 accumulated 15-ADON, and M9-07-1/Carman-705-2 produced 3-ADON in the infected spikes of AC Nanda (a soft white spring wheat variety).

at various intervals after fungal inoculation in spikes of AC Nanda, a FHB susceptible wheat variety. Mycotoxin act as virulent factors for fungal growth and disease progression therefore, concentrations of DON ($r = 0.659$, $P \leq 0.001$) and 3-ADON ($r = 0.439$, $P \leq 0.01$) showed a significant positive correlation with FHB severity. D3G being a glucoside of DON also had a significant positive correlation ($r = 0.597$, $P \leq 0.001$) with disease severity. DON showed significant positive correlation both with D3G ($r = 0.931$, $P \leq 0.001$) and 3-ADON ($r = 0.5$, $P \leq 0.05$) concentrations. NIV and 15-ADON did not correlate significantly with the FHB severity and other mycotoxin accumulation due to their absence or trace concentrations in spikes infected with all *F. graminearum* isolates.

6.4.3 Spike culture derived variants varied significantly for FHB disease severity

The 55 SCDV genotypes differed significantly in FHB severity when infected with the five different *F. graminearum* isolates. The spikes infected with 3-ADON producing isolates (22.5-100.0% with an average of 45.1%, 28.9-100.0% with an average of 66.2%, 33.4-100.0% with an average of 77.6%, and 60.0-100.0% with an average of 92.9% at 5, 7, 9 and 11 DAI) had a higher FHB severity compared to those infected with 15-ADON (11.6-100.0% with an average of 38.7%, 18.6- 100.0% with an average of 56.8%, 32.7-100.0% with an average of 72.7%, and 43.3-100.0% with an average of 92.2% at 5, 7, 9 and 11 DAI) and NIV (13.7-100.0% with an average of 30.9%, 19.6-100.0% with an average of 48.9%, 31.6-100.0% with an average of 68.3%, and 57.5-100.0% with an average of 91.6% at 5, 7, 9 and 11 DAI) producing chemotypes (Figure 6.3). Wheat SCDV genotypes and *F. graminearum* isolates (chemotypes) showed significant effect on FHB severity at 5 [Mean sum of squares (MSS) = 0.161 and 0.453 at $P \leq 0.001$], 7 (MSS = 0.211 and 0.674 at $P \leq 0.001$), and 9 (MSS = 0.169 and 0.207 at $P \leq 0.001$) DAI whereas at 11 DAI, the effect of wheat genotypes (MSS = 0.049 at $P \leq 0.001$) was significant but non-significant for chemotypes (MSS = 0.037 at $P > 0.05$).

6.5 Discussion

The present study documented the co-occurrence of DON, and its acetylated forms in wheat spikes which is more toxic to human health than predicted from mycotoxin present individually (Alassane‐Kpembi et al., 2015; Del Regno et al., 2015; Ferrigo et al., 2016;

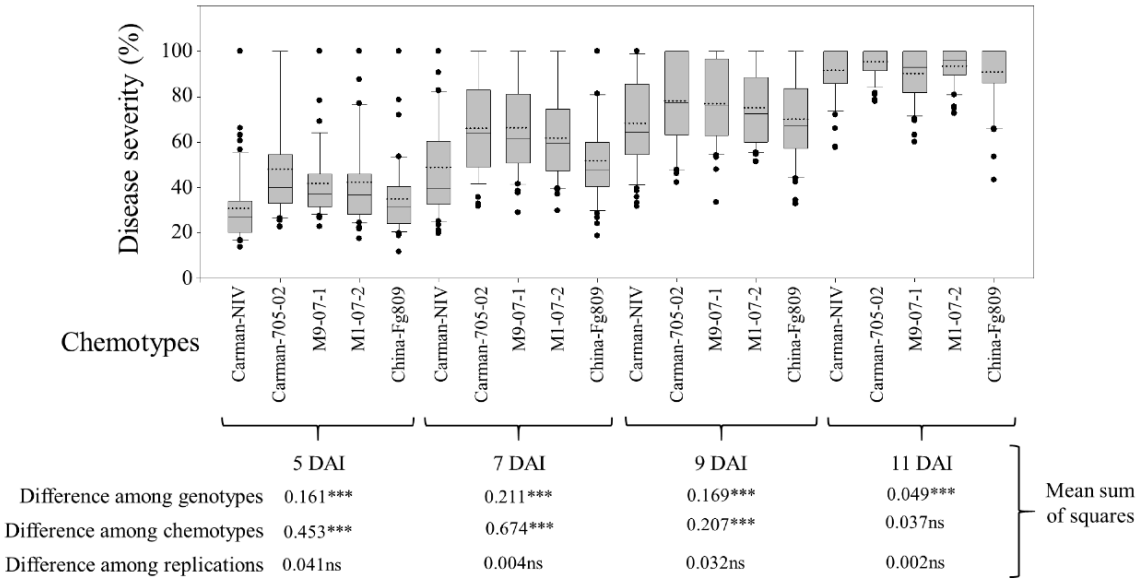


Figure 6.3: Boxplot analysis summarizing *Fusarium* head blight (FHB) severity at 5, 7, 9 and 11 days after inoculation (DAI) in the spikes of 55 spike culture derived wheat variants infected with *F. graminearum* isolates (M9-07-1, M1-07-2, Carman-NIV, China-Fg809, and Carman-705-2) along with Mean Sum of Square values obtained from Analysis of Variance using General Linear Model showing differences for FHB severity among wheat genotypes (SCDV), isolates and replications. Dotted line in box represent average of the disease severity for the corresponding dataset whereas, *** and ns stand for significant difference at $P \leq 0.001$ and non-significant, respectively.

van der Lee et al., 2015). The correlation between FHB severity and mycotoxin accumulation is also influenced by the wheat genotypes infected. A significant positive (Bai et al., 2001; Homdork et al., 2000) as well as no correlation (Chen et al., 1996; Miller et al., 1985) have been reported between the two factors in previous studies (Ji et al., 2015). The higher aggressiveness of 3-ADON producing isolates concurred with the conclusions of Puri and Zhong (2010); however, von der Ohe et al. (2010) aDnd Liu et al. (2017) did not find any significant difference among distinct chemotypes of *F. graminearum* for aggressiveness but observed higher accumulation of DON in grains infected with 3-ADON producing isolates. Therefore, aggressive of a strain rather than being determined merely by the mycotoxin(s) produced, is also influenced by its genome, environment and its interaction with host wheat genotype (Howlett et al., 2015; Pariaud et al., 2009).

Out of 55 SCDV lines, five showed resistance against multiple isolatesof *F. graminearum* (Table 6.1). FHB severity was observed at 5, 7, 9 and 11 DAI however, only at 7 and 9 DAI was compared. FHB severity at 5 and 11 DAI was too low and high, respectively, to be useful to identify FHB resistant lines. At 7 and 9 DAI, AC Nanda (FHB susceptible control) FHB disease severity varied from 42.6 to 65.3% and 59.9 to 90.7%, whereas it ranged from 26.6 to 59.8% and from 40.7 to 59.8% in Sumai-3 (FHB resistant control). SCDV lines FHB213.4, FHB244.1, FHB245.6, FHB250.2, and FHB252.3 showed resistance to multiple *F. graminearum* isolates among which FHB severity ranged between 20.6-45.5/38.6-63.6%, 32.5-48.4/43.7-52.3%, 31.7-50.3/47.7-64.3%, 29.7-50.9/54.4-86.0%, and 30.5-46.3/43.9-56.1% at 7/9 DAI, respectively. The selected SCDV lines showed even better FHB resistance compared to Sumai-3 for some *F. graminearum* isolates. These selected SCDV lines could be utilized in a breeding program to introgress FHB resistance against myltiple isolates in to elite wheat varieties.

Table 6.1: Comparison of Fusarium head blight (FHB) at 7 and 9 days after inoculation among the selected spike culture derived variants, AC Nanda (FHB susceptible control) and Sumai-3 (FHB resistant control)

Chemotypes	Days after inoculation	FHB Severity (%)						
		Spike Culture Derived Variants					AC Nanda	Sumai-3
		FHB213.4	FHB244.1	FHB245.6	FHB250.2	FHB252.3		
M1-07-2	7	30.7 ± 2.1	48.4 ± 8.2	39.6 ± 0.6	29.7 ± 2.7	46.3 ± 1.5	50.4 ± 2.1	35.9 ± 1.7
	9	44.9 ± 1.4	51.2 ± 4.2	51.4 ± 5.1	54.4 ± 1.7	53.3 ± 0.9	65.3 ± 1.0	40.7 ± 2.2
M9-07-1	7	45.5 ± 11.9	40.0 ± 11.3	45.5 ± 9.4	42.5 ± 11.1	31.8 ± 4.6	65.3 ± 10.3	59.8 ± 2.3
	9	63.6 ± 9.3	44.0 ± 5.7	55.4 ± 7.7	55.6 ± 4.9	56.1 ± 1.5	90.7 ± 13.1	59.8 ± 2.3
Carman-NIV	7	20.6 ± 7.8	37.3 ± 4.8	50.3 ± 12.3	50.9 ± 7.1	39.5 ± 1.7	42.6 ± 4.9	35.1 ± 2.5
	9	38.6 ± 5.1	52.3 ± 3.9	64.3 ± 3.4	86.0 ± 0.5	48.3 ± 4.7	60.3 ± 5.3	42.6 ± 3.4
China-Fg809	7	33.9 ± 7.5	32.5 ± 5.5	32.4 ± 8.9	34.8 ± 12.3	30.5 ± 1.9	49.0 ± 7.8	34.5 ± 3.4
	9	47.3 ± 0.8	43.7 ± 1.2	64.3 ± 5.1	55.9 ± 5.2	43.9 ± 0.9	59.9 ± 1.3	44.6 ± 0.7
Carman-705-2	7	44.0 ± 0.0	45.0 ± 8.7	31.7 ± 4.4	48.6 ± 2.0	36.6 ± 2.2	65.3 ± 3.9	26.6 ± 3.6
	9	46.0 ± 2.8	49.9 ± 4.8	47.7 ± 0.2	55.1 ± 0.2	47.3 ± 3.2	73.9 ± 6.0	44.6 ± 0.8

CHAPTER 7. CORRELATION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN *UDP-GLUCOSYLTRANSFERASE* GENE WITH FUSARIUM HEAD BLIGHT TYPE II RESISTANCE IN F_2 PROGENY

Study 5

The objective of this study was to use the *TaUGT-3B* SNP to follow the FHB resistance in a back cross experiment.

CH conducted the DRM screening of the progeny.

Huang, C., Gangola, M. P., Hucl, P. and Chibbar, R. N. Correlation of single nucleotide polymorphisms (SNP) of *UDP-glucosyltransferase* gene with Fusarium head blight type II resistance in BC_2 progeny using high resolution melting curves to detect SNPs. (to be submitted).

7.1 Abstract

Four FHB resistant SCDV genotypes (FHB202, FHB244, FHB250 and FHB256) were crossed with an elite wheat cultivar CDC Hughes (PT588). Four F_2 and four BC_1F_2 progeny populations generated by crossing PT588 and FHB resistant wheat genotypes were assessed for the *TaUGT-3B* SNP by high resolution melt curve analysis. The resistant allele frequency in FHB202/PT588, FHB244/PT588, FHB250/PT588 and FHB256/PT588 was 5.9%, 9.1%, 34.6% and 10.5%, with a mean value of 15.0% for the respective populations. No FHB resistant lines were observed in PT588*2/FHB202 and PT588*2/FHB244, but 17.4% and 27.2% were observed in PT588*2/FHB250 and PT588*2/FHB256, with an average frequency of 11.17%. The association of genotypic results determined by high resolution melt curve with phenotypic data assessed as FHB severity was assessed by analysis of variance (ANOVA). The FHB severity of maker carriers and non-carriers differed significantly ($P<0.01$).

7.2 Introduction

Development of wheat cultivars with durable FHB resistance is one of the strategies to control FHB infection (Beres et al., 2018). Backcrossing (BC) is one of the widely adopted approaches in wheat to introgress biotic resistance from the donor parent in to recurrent parent (Rai et al., 2018). The recurrent parent in a backcross refers to an elite cultivar having most of the desirable traits but devoid of only few characteristics that will be incorporated from the donor parent through backcrossing (Collard and Mackill, 2008). Traditional backcrossing requires about six backcrosses to obtain about 99% recurrent parental genome recovery which is a time- and labor- intensive approach. Theoretically, BC_1 progeny on an average contain 75% of the recurrent parent genome however, some individuals contain higher proportions. Such individuals can be identified by utilizing recurrent parent specific molecular markers in a backcross breeding program (Hasan et al., 2015; Rai et al., 2018). The integration of molecular markers associated with the desirable trait and specific to a recurrent parent's genome in backcrossing can identify individuals with the desirable trait and >99% of the recurrent parent's genome even in BC_2 or BC_3 progeny thus accelerating the process of cultivar development. Therefore, molecular marker assisted backcross breeding was utilized in the present project in which SCDV lines

(FHB202, FHB244, FHB250 and FHB256) with stable FHB resistance and a wheat cultivar, CDC Hughes (a Canada Western Red Spring wheat line developed by Dr. Piere Hucl, University of Saskatchewan) were utilized as donor and recurrent parents, respectively whereas the SNP marker on *TaUGT-3B* strongly associated with FHB resistance acted as the molecular marker to enhance the selection of progenies. The correlation analysis showed a significant correlation between SNP on *TaUGT-3B* and FHB reaction in the backcrossed progeny.

7.3 Materials and methods

7.3.1 The generation of BC_1F_2 progeny from different cross combinations and plant material for the characterization of the progeny

Four FHB resistant SCDV genotypes (FHB202, FHB244, FHB250 and FHB256) characterized in this project were crossed with CDC Hughes. The F_2 as well as the BC_1F_2 progeny was analyzed for the *TaUGT-3B* SNP and FHB reaction by immature spike culture (Sharma et al., 2018). The results showed that FHB resistance was associated with the *TaUGT-3B* SNP and both were transferred to the progeny.

7.3.2 Assessment of FHB resistance using immature spike culture

Three spikes were cut at heading stage from each of the total 191 wheat plants. After four days of culture in spike culture media, on the fifth day, the wheat spikes were inoculated with Fusarium M7 strain as described (Chapter 3). After inoculation, the plants were bagged with plastic bags and placed in an incubator as described (Chapter 3). After 2.5 days of incubation, the bags were removed, and on the eighth day after inoculation, the first rating of disease severity rating was done as described (Sharma et al., 2018). The second, third, and fourth disease ratings were conducted on every other day.

7.3.3 DNA extraction procedure and high resolution melt curve (HRM) to identify plants carrying the *TaUGT-3B* SNP

The 191 leaf samples stored at -80 ℃ freezer were used for DNA extraction. The half part from each leaf was squashed by silicon beads placed in 2 mL tubes by a vibration machine under -20 ℃ (Precellys 24, Bertin technologies, France). A commercial kit

(Qiagen DNeasy Plant Mini Kit, Qiagen, Germantown, MD) was used to extract DNA following the manufacturer's protocol. The isolated DNA was used for high resolution melting curve (HRM) analysis (Chapter 3). First a pair of primers was used in nested PCR to amplify the genes targeting the SNP of *TaUGT-3* at 1558 bp from start codon. The amplified PCR fragment was used in the HRM analysis. Sumai-3 and AC Nanda were used as control in all the HRM analysis experiments. The DNA sequences of this gene and information of primers (Table 3.1) and conditions were as described Sharma et al. (2018).

The total volume of 20 µL for HRM reaction contained 10 µL of HRM super mix from Precison Melt Supermix (Bio-Rad laboratories, Inc., USA), 10 µM of each pair of primers, 1.0 µL PCR products from the first round regular PCR as DNA template and sterile deionized water. The HRM analysis was done in two consecutive steps in a CFX96TM Real-Time System (Bio-Rad Laboratories (Canada) Ltd.). The two steps included the amplification and melting curve assays. The amplification step included a 10 min initial denaturation, then the 40 cycles of 95°C for 15 s and 60°C for 30 s. The HRM step consisted of denaturation for 10 s at 95 ℃, the annealing of 1 min at 60 ℃, high resolution melting stage for 15 s at 95 ℃, followed by the final annealing step for 1 min at 60 ℃. The temperature increased from 60 ℃ to 95 ℃ was set in increments of 0.1 ℃ with a holding time of 10 s at each increment. The high resolution melting curve results were analyzed by Bio-Rad CFX Manager 3.1 and Bio-Rad Precision Melt Analysis software (Bio-Rad Laboratories, Canada). The SCDV variants tested for nucleotide variation were readily differentiated by the melting curve plot of fluorescent difference of different genotypes.

7.4 Results

7.4.1 The summary of genotyping results of different wheat crossing combinations

A total of 191 wheat plants from eight cross combinations, with varying number of plants derived from each combination, were analyzed for SNP in *TaUGT3* by real-time PCR and HRM analysis. In the first four crosses, the four selected resistant SCDV wheat lines were the female parent, while the common wheat cultivar CDC Hughes was the male parent. In the last four crosses (BC_1), the female parent was CDC Hughes, while F_1 plants were the male parents (Figure 7.1). The progeny from the BC_1F_2's showed SNP variation which distinguished between the homozygotes and heterozygotes (Figure 7.2).

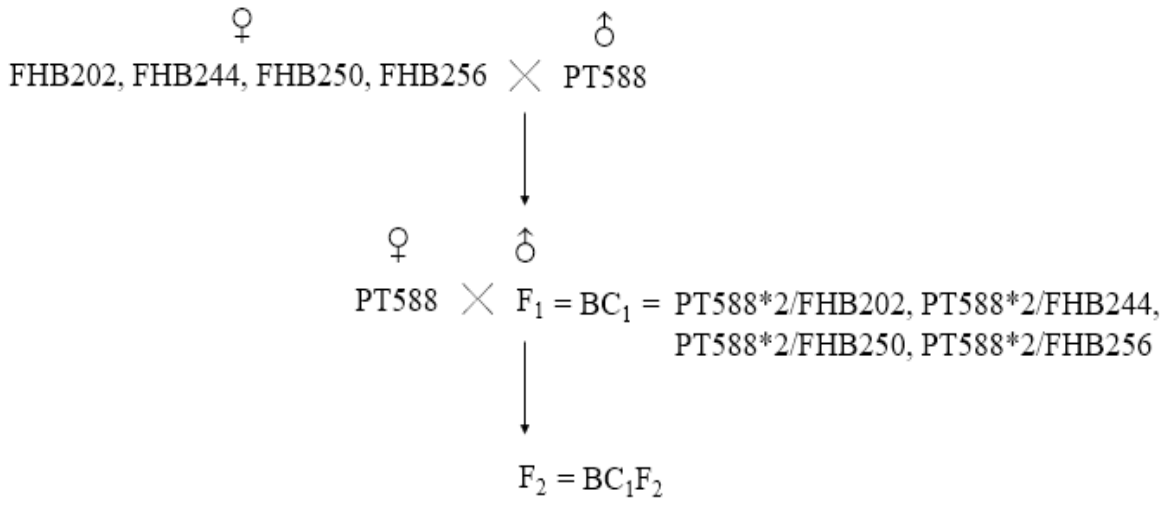


Figure 7.1: Diagram of different cross combinations used in this study. The seeds of progeny population in BC_2 generation of eight cross combinations between resistant SCDV wheat genotypes and elite wheat cultivar PT588 were used for assessing FHB disease severity and *TaUGT-3B* SNP using HRM analysis.

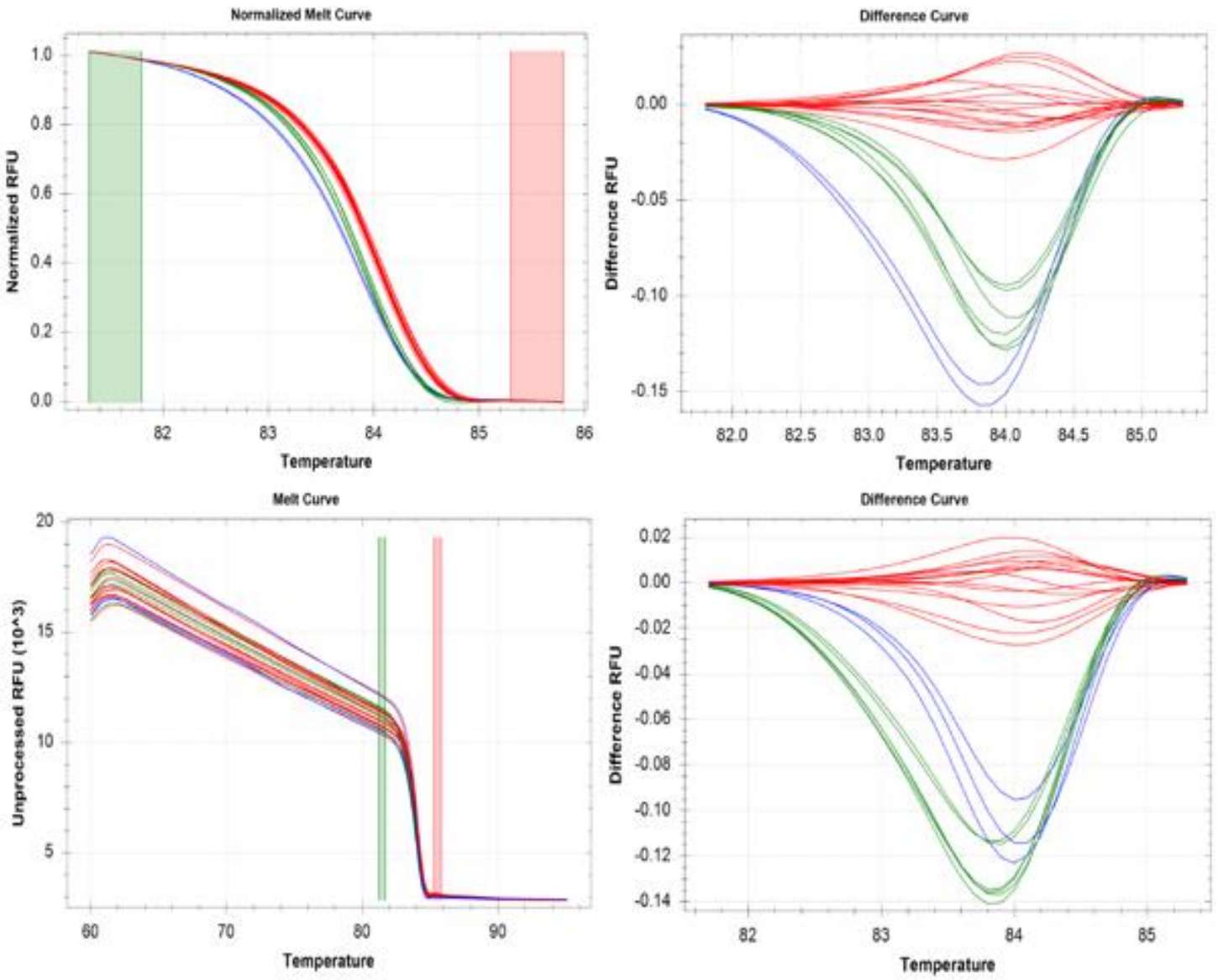


Figure 7.2: High resolution melt (HRM) curves of BC_2 progeny from wheat population in different cross combinations between resistant wheat genotypes and elite wheat cultivar PT588, contrasting wild, heterozygous, and homozygous genotypes of SNP in gene *TaUGT3B*.

The frequencies of homozygotes, heterozygotes and wild type of SNP in gene *TaUGT3B* are summarized in Table 7.1. The highest frequency of the homozygous mutation type, which is the same as present in Sumai-3, was detected in FHB250/PT588, with nine homozygotes out of 26 plants tested, corresponding to 34.6%. The lowest frequency of homozygotes was detected in FHB202/PT588, with only one homozygote, accounting for 5.9% of the F_2 population. In FHB244/PT588 and FHB256/PT588, both crosses had two homozygotes with the same SNP type as Sumai-3, accounting for 9.0% and 10.5%, respectively, of the progeny. The highest frequency of heterozygotes was detected in FHB256/PT588, with 11 (57.89%) being identified, followed by FHB250/PT588, with 12 (46.15%), then FHB202/PT588 (23.53%), and FHB244/PT588 (22.73%). The four BC_1F_2 populations had lower (PT588*2/FHB244, PT588*2/FHB250, PT588*2/FHB256) or similar (PT588*2/FHB202) heterozygote frequencies compared to the F_2 populations, with percentages of 17.86%, 17.39%, 13.64%, and 23.53%, respectively. There were no homozygote carriers identified in PT588*2/FHB202 and PT588*2/FHB244. The highest rate of carrier homozygotes was observed in PT588*2/FHB256 (27.27%), followed by PT588*2/FHB250 (17.39%) (Table 7.1).

7.4.2 FHB disease severity frequency distribution of progeny in eight different cross combinations

To study the efficiency of molecular screening by HRM for SNP, FHB disease severity was assessed by immature spike culture screening. Genotyping results showed that the wheat plants could be categorized as wild type (same as AC Nanda), heterogygotes, and homozygotes (mutant type - same as Sumai-3). The distribution frequency of FHB disease severity in all the 62 spikes collected in the FHB202/PT588 population, the highest frequency occured between 60%-70%, with 14 spikes being recorded; followed by 50%-60% and 70%-80%, with 12 spikes of each. A similar situation occurred in the populations derived from FHB244/PT588, FHB250/PT588, and FHB256/PT588, with the highest severity frequency between 50%-60%, 70%-80%, and 60%-70%, out of total 97, 112, and 65 spikes, respectively (Figure 7.3). In the F_2 populations PT588*2/FHB202, PT588*2/FHB244, PT588*2/FHB250, and PT588*2/FHB256, 86, 71, 77 and 52 spikes were collected and recorded with disease severity on 9 DAI, respectively (Figure 7.4).

Table 7.1: The summary of percentages of wild, heterozygotes, and homozygotes for SNP in gene *UGT3* in the progeny population (3 leaf samples as replicates mixed for one plant) from different cross combinations between elite wheat cultivar PT588 and FHB resistant SCDV genotypes FHB202, FHB244, FHB250 and FHB256, by HRM genotyping analysis.

Number of Cross combinations	Total # of progeny plants	Generation	Wild type progeny plants (AC Nanda) (%)	Homozygotes mutation - # progeny plants (%)	Heterozygotes mutation - # progeny plants (%)
FHB202/PT588	17	F_2	12 (70.6)	1 (5.9)	4 (23.5)
FHB244/PT588	22	F_2	15 (68.2)	2 (9.1)	5 (22.7)
FHB250/PT588	26	F_2	5 (19.2)	9 (34.6)	12 (46.2)
FHB256/PT588	19	F_2	6 (31.6)	2 (10.5)	11 (57.9)
Total #	84	F_2	38	14	32
PT588*2/FHB202	34	BC_1F_2	26 (76.5)	0 (0.0)	8 (23.5)
PT588*2/FHB244	28	BC_1F_2	23 (82.1)	0 (0.0)	5 (17.9)
PT588*2/FHB250	23	BC_1F_2	15 (65.2)	4 (17.4)	4 (17.4)
PT588*2/FHB256	22	BC_1F_2	13 (59.1)	6 (27.3)	3 (13.6)
Total #	107	BC_1F_2	77	10	20
Combined Total #	191				

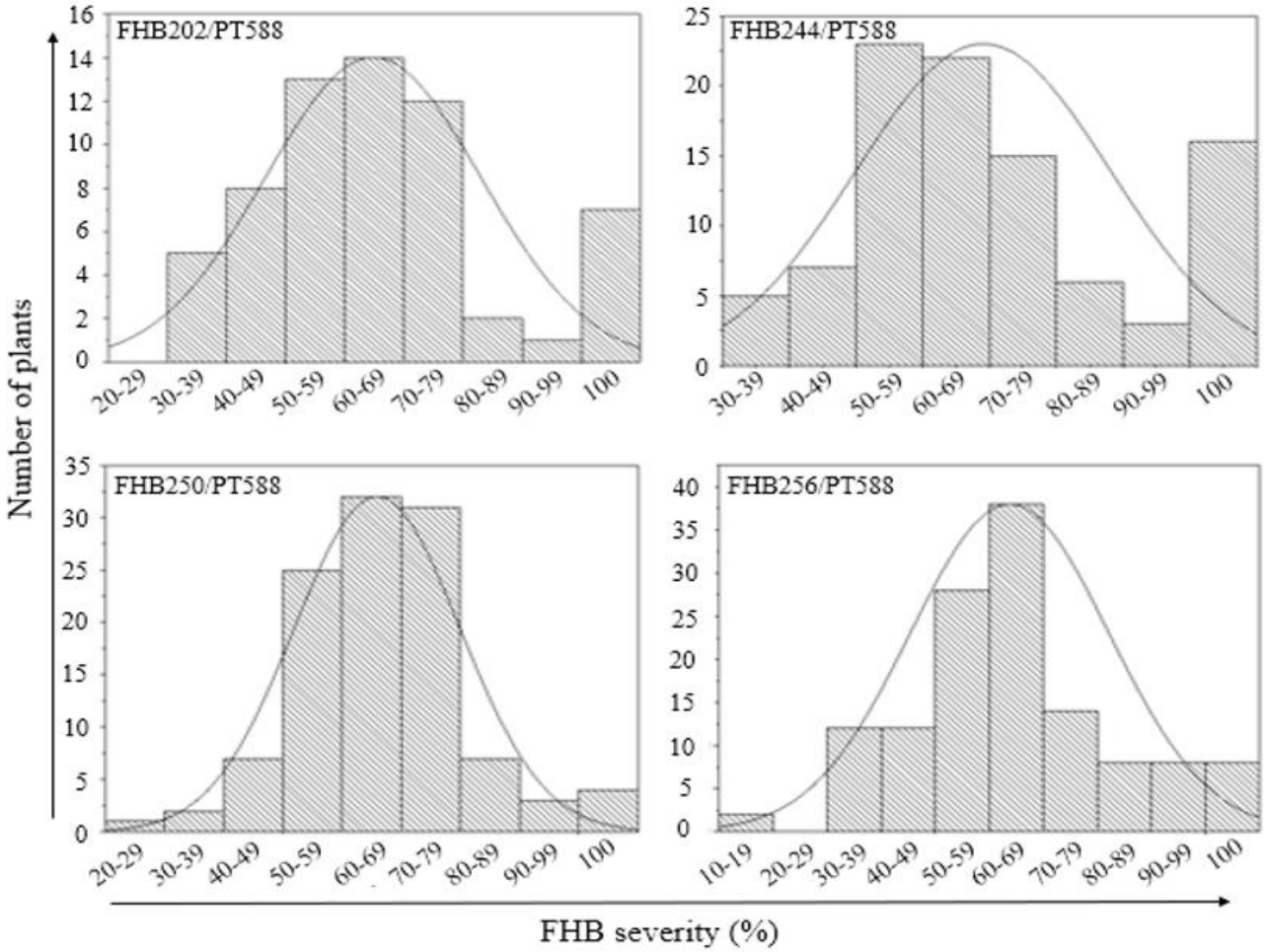


Figure 7.3: The frequency distribution of FHB severity on 9 Days after inoculation (DAI) of F_2 progenies from wheat population of different cross combinations between resistant wheat genotypes FHB202, FHB244, FHB250, FHB256 and elite wheat cultivar PT588. Three replicates were observed and recorded for each plant in this phenotyping analysis.

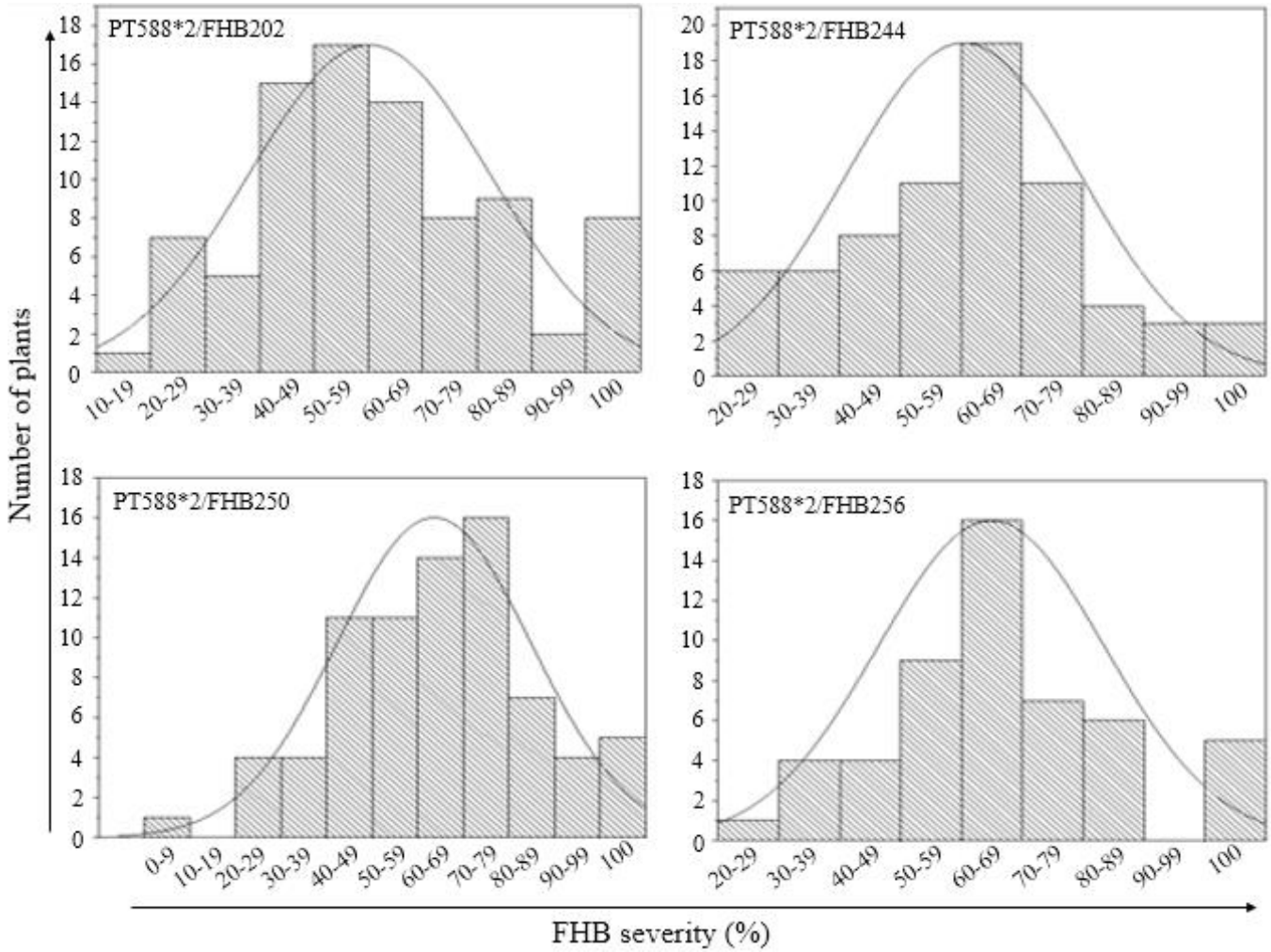


Figure 7.4: The frequency distribution of FHB severity on 9 Days after inoculation (DAI) of BC_1F_2 progenies from wheat population of different crossing combinations between elite wheat cultivar PT588 and resistant wheat genotypes FHB202, FHB244, FHB250, FHB256.

For PT588*2/FHB244 and PT588*2/FHB256, the highest frequencies were both between 60%-70%, in PT588*2/FHB202 was between 50%-60%, while in PT588*2/FHB250 was from 70% to 80% (Figure 7.4).

7.4.3 Analysis of variance (ANOVA) of Fusarium head blight severity of eight different cross combinations

In the cross FHB202/PT588, on 9 DAI, the disease severity of resistant genotypes varied from 37% to 48%, with mean value of 43% ± 5%, showing similar level to Sumai-3, which ranged from 36% to 44%, with average of 40% ± 4% (Figure 7.5). These two categories showed statistically significantly lower values of FHB severity when compared with susceptible genotypes and AC Nanda, ranging from 30% to 100%, and from 78% to 84%, respectively, with mean values of 69% ± 19%, and 81% ± 3%. The heterozygotes ranked midway between these two categories and showed non-significant differences from both of these two categories, varying from 31% to 90%, with average of 61% ± 14%. In the combination of FHB244/PT588, the analysis of variance (ANOVA) result of disease severity was similar to that of FHB202/PT588. In the cross combinations FHB250/PT588 and FHB256/PT588, there were no significant differences between categories of Sumai-3 and homozygous SNP-carrier genotypes (same as Sumai-3), as well as between homozygous non-carrier genotypes (same as AC Nanda) and AC Nanda; however, the severity results from homozygous SNP carriers and Sumai-3 were significantly lower (P=0.05) than homozygous non-carriers and AC Nanda. The heterozygotes genotypes were in between of carrier- and non-carrier- categories, showing non-significant differences from both categories (Figure 7.5). In the cross combinations PT588*2/FHB202 and PT588*2/FHB244 on 9 DAI (Figure 7.6), the homozygous carriers were not detected, however, the heterozygote genotypes in both populations showed the same FHB resistance level as Sumai-3, which was statistically significantly lower than that of the non-carrier lines and AC Nanda. In the cross combinations PT588*2/FHB250 and PT588*2/FHB256 (Figure 7.6), the SNP carriers showed the same level of disease severity as Sumai-3, which was significantly lower than that of non-carriers and AC Nanda. On the other hand, the FHB severity of heterozygotes was in between the two homozygotes, showing no statistically significant difference between those two genotypes.

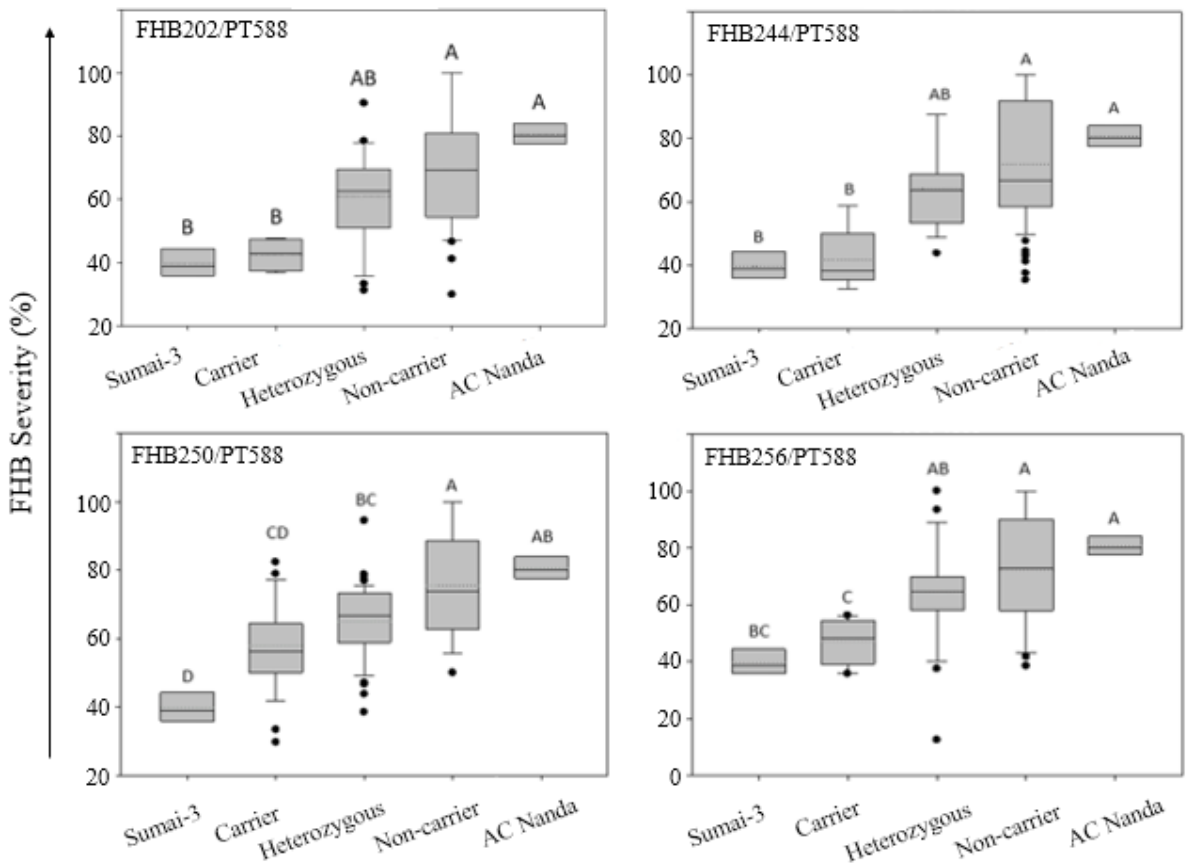


Figure 7.5: The ANOVA results of FHB severity of different genotypic categories determined by HRM: Sumai-3, homozygous carriers (the mutant type of SNP, same as Sumai-3), heterozygotes genotypes, non-carriers (the wild type of SNP, same as AC Nanda) and AC Nanda on 9 Days after inoculation (DAI) of F_2 progeny from wheat population of different cross combinations between resistant wheat genotypes FHB202, FHB244, FHB250, FHB256 and elite wheat cultivar PT588. Different letters showing statistically significant differences of multiple levels of FHB severity ($P<0.01$).

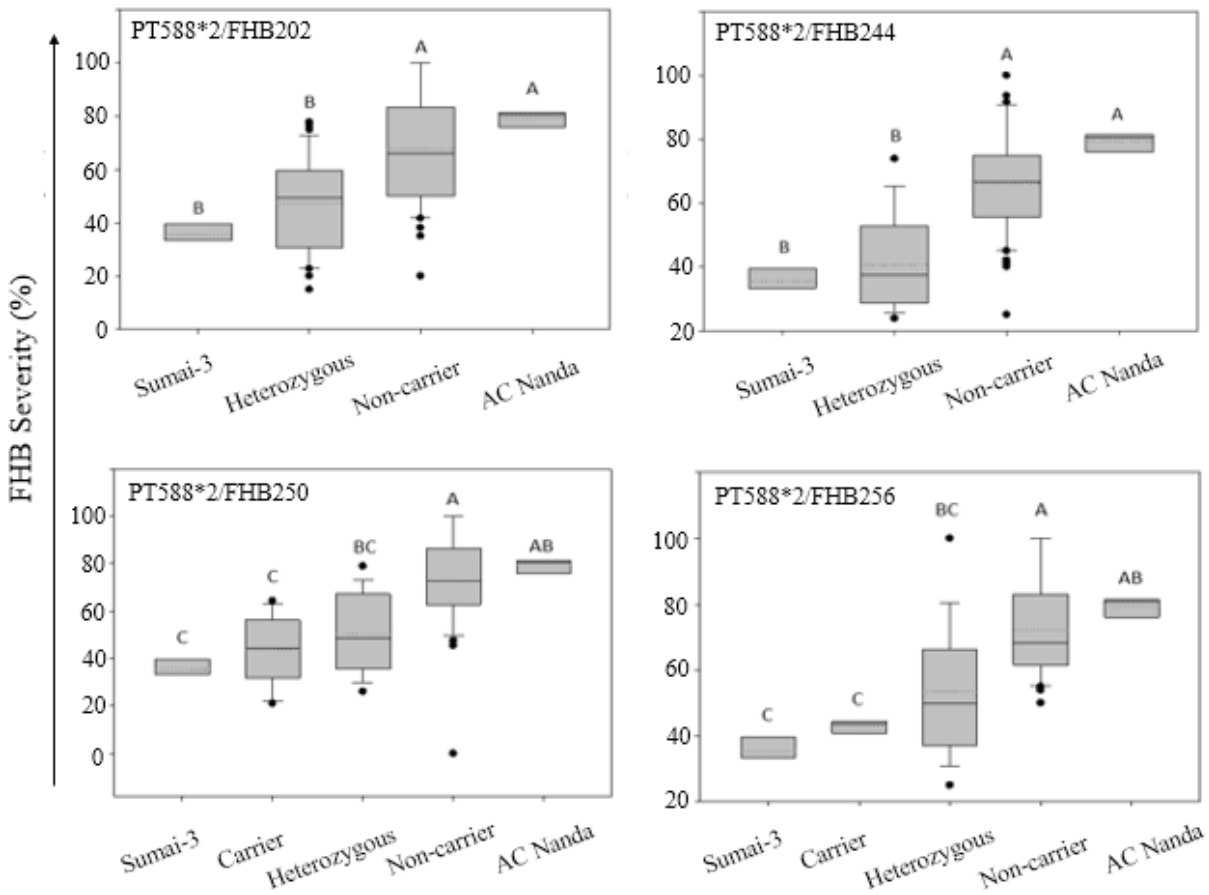


Figure 7.6: The ANOVA results for FHB severity of different genotypic categories determined by HRM: Sumai-3, homozygous carrier (the mutant type of SNP, same as Sumai-3), heterozygous genotypes, non-carrier (the wild type of SNP, same as AC Nanda) and AC Nanda on 9 Days after inoculation (DAI) of BC_1F_2 progenies from wheat population of different crossing combinations between elite wheat cultivar PT588 and resistant wheat genotypes FHB202, FHB244, FHB250, FHB256. Different letters showing statistically significant differences of multiple levels of FHB severity ($P<0.01$).

The variations of FHB severity of progeny from these eight combinations on 5, 7, 9, 11 DAI were showed in Figure 7.7 and Figure 7.8. In each of these combinations, the gradual increase of FHB severity from 5 to 11 DAI was observed; although different resistant parents resulted in slightly different levels of FHB severity on the same day. However, the FHB severity of Sumai-3 and homozygote carriers were consistently lower than that of the heterozygotes.

7.5 Discussion

Marker assisted backcross breeding has two major selection procedures: (i) foreground selection which utilizes molecular markers associated with the target gene, and (ii) background selection consisting of molecular markers flanking the target gene/region and associated with other non-target regions in the recurrent parent' genome (Semagn et al., 2006). The present experiment represents the foreground selection of individuals confirming the introgression of SNP on *TaUGT-3B* in to the backcross progenies. The SNP on *TaUGT-3B* has been identified as a molecular marker strongly associated FHB resistance in SCDV lines (Sharma et al., 2018) therefore, showed a significant impact on FHB resistance. The individuals with homozygous allele like Sumai-3 (FHB resistant control genotype) showed significantly reduced FHB severity compared to the individuals with homozygous allele like AC Nanda (FHB susceptible control genotype) and heterozygous alleles (Figures 7.5 and 7.6). The individuals with homozygous Sumai-3 like alleles will be evaluated for the background selection to identify individual(s) with maximum proportion of recurrent parent's genome.

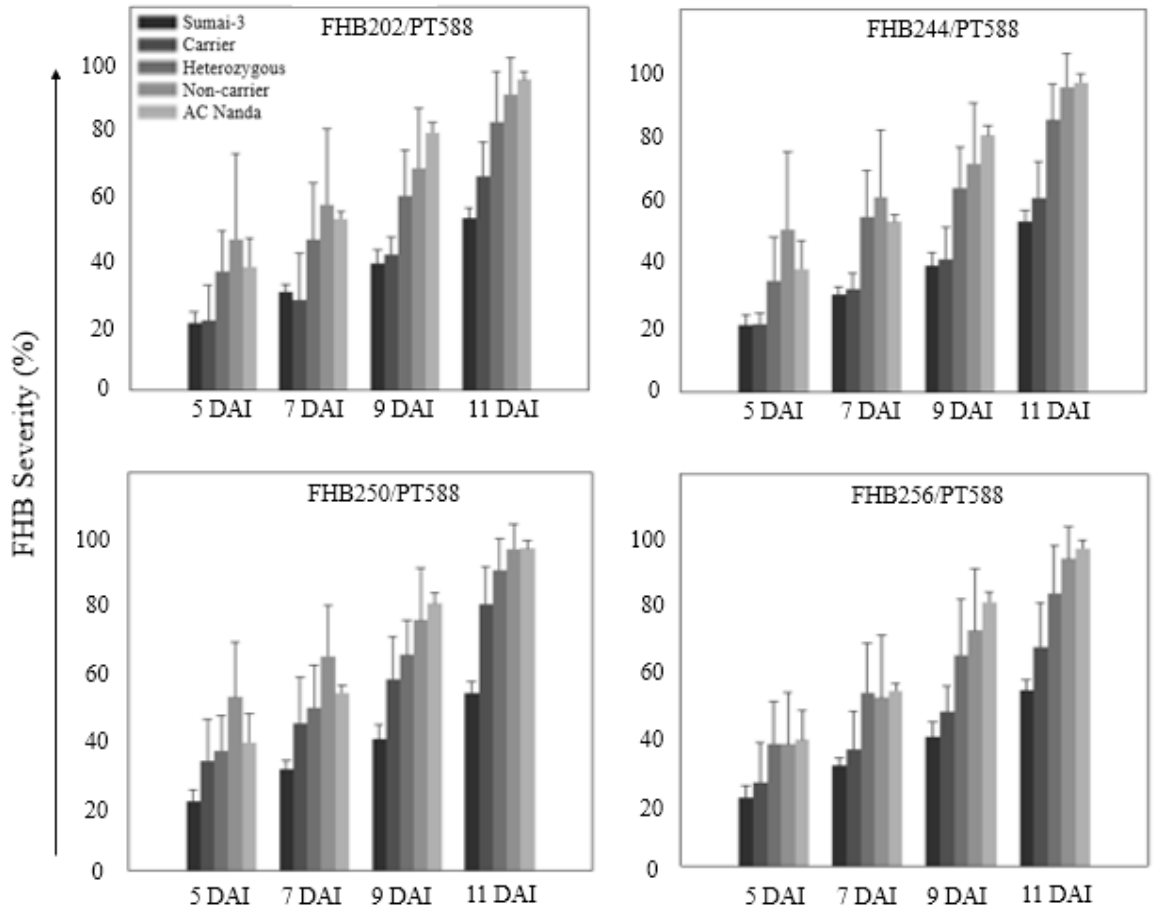


Figure 7.7: The FHB severity values (mean ± std. dev.) of different genotypic categories determined by HRM: Sumai-3, homozygous carrier (the mutant type of SNP, same as Sumai-3), heterozygous genotype, non-carrier (the wild type of SNP, same as AC Nanda) and AC Nanda on 9 Days after inoculation (DAI) of F_2 progeny from wheat populations of different cross combinations between resistant wheat genotypes FHB202, FHB244, FHB250, FHB256 and elite wheat cultivar PT588.

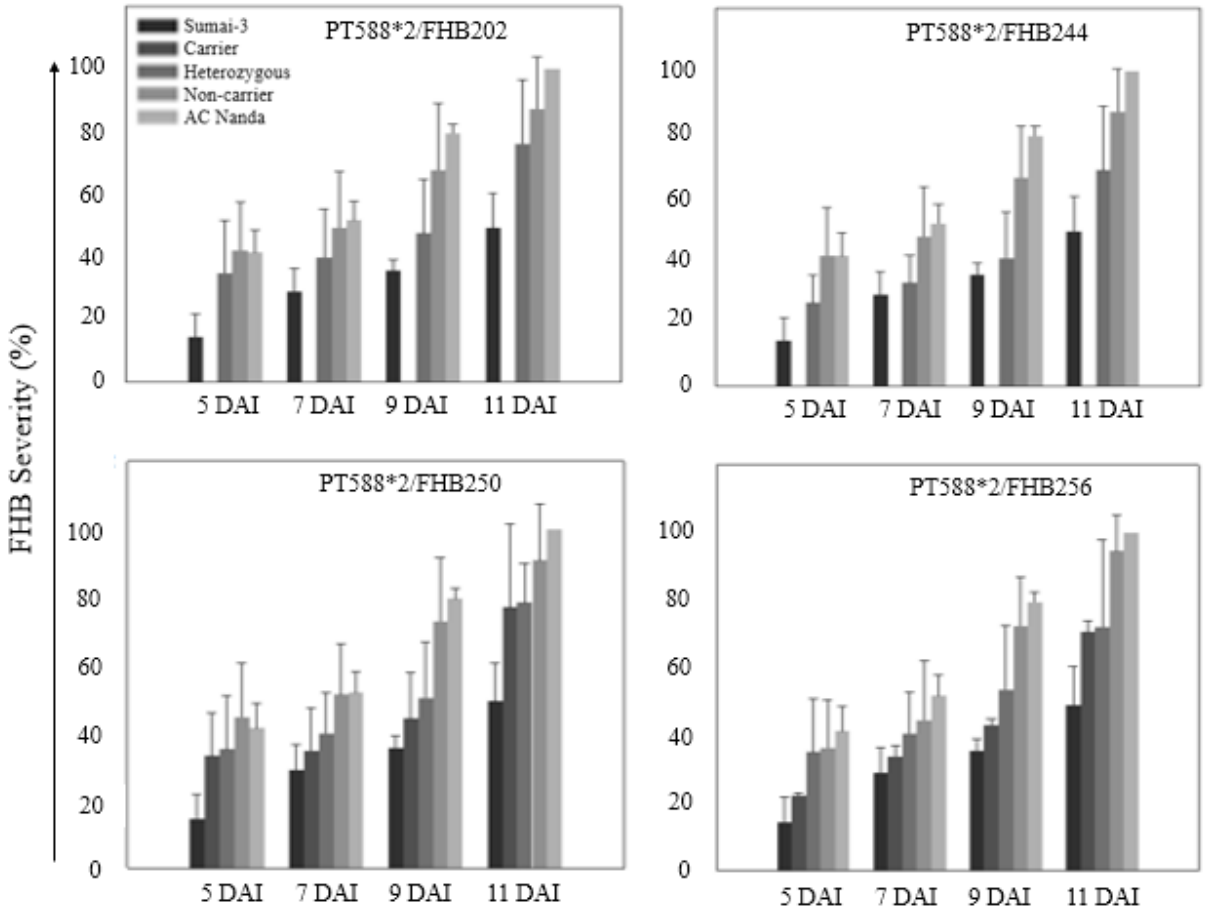


Figure 7.8: The FHB severity (mean ± std. dev.) of different genotypic categories determined by HRM: Sumai-3, homozygous carrier (the mutant type of SNP, same as Sumai-3), heterozygous genotype, non-carrier (the wild type of SNP, same as AC Nanda) and AC Nanda on 9 DAI of BC_1F_2 progeny derived from FHB resistant wheat genotypes FHB202, FHB244, FHB250, FHB256.

CHAPTER 8. GENERAL DISCUSSION AND CONCLUSIONS

8.1 Inferences

Fusarium Head Blight (FHB) is a devastating disease of wheat, which in recent years has been gradually spreading from east to west on the Canadian prairies (Brar et al., 2016). FHB resistant wheat varieties can play a major role in mitigating the devastating effects of FHB. Wheat varieties with moderate to low FHB resistance have been developed (Beres et al., 2018). To develop FHB resistant varieties there is a need to identify new sources of FHB resistance, develop a rapid FHB screening technique to identify FHB resistant germplasm and finally study the relationship between FHB disease severity and mycotoxin production in the grain.

8.1.1 *In vitro* immature spike cultures can be used to screen FHB resistance

There are three widely accepted types of FHB resistance (Bai and Shaner, 1994). According to Schroeder and Christensen (1963), Type I resistance is the resistance to initial infection by the pathogen; and the resistance to FHB symptoms spread within a spike is termed as Type II resistance. Miller et al. (1985) introduced the Type III resistance concept, which is described as a treduced level of mycotoxin accumulation. Type II resistance is the most stable resistance therefore most easily utilized in wheat breeding programs (Bai and Shaner, 2004; Bai et al., 2018). Type II resistance is assessed by the injection of inoculum into a central spikelet in the greenhouse or the spread of grain-spawn inoculation in field (Bai and Shaner, 1994; Bai et al., 2018). Seceral different strategies are used to assess FHB resistance in the field. To accurately assess FHB severity (type II resistance), the central spikelet of a spike is usually point-inoculated with the inoculum. The percentage of symptomatic spikelets of a spike (PSS) or a visual FHB scale from 1 to 10 (Stack and McMullen, 1995) is used to represent disease severity (Bai and Shaner, 1994; Jin et al., 2013). *F. graminearum* spore suspension is also used in conjugation with corn spawn. In some nurseries as in Carman, Manitoba, a mist irrigation system is applied in combination with the spore suspension and corn spawn, to optimize the conditions for FHB disease development.

Before applying the *in vitro* spike culture screening method to the SCDV population,

50 AC Nanda (the susceptible control) and 50 Sumai-3 (the resistant control) were evaluated by *in vitro* spike culture, to examine the feasibility of differentiating between resistant and susceptible genotypes, by using two phenotyping approaches (Figure 3.4). Both the methods PSS (Bai and Shaner, 1994) and visual rating scale (Stack and McMullen, 1995) distinguished the resistant (Sumai 3) and susceptible (AC Nanda) genotypes (Figure 3.4). Oliver et al. (2008) used Sumai-3 and "Russ" as resistant and susceptible controls, respectively, in greenhouse (three runs) and field evaluations (two locations) to screen for FHB severity of wheat accessions. The PSS based FHB severity of Sumai-3 varied from 6.32 to 12.02 through three greenhouse runs after three weeks of inoculation; while the susceptible control Russ ranged from 28.40 to 79.04. In field nurseries, FHB severity of Sumai-3 varied from 0.64 to 13.16 across two locations; while Russ averaged from 25.04 to 75.64. Jin et al. (2013) also used Sumai-3 as a resistant genotype in both greenhouse and field tests to assess the FHB severity of wheat accessions. In their study, the PSS based FHB severity of Sumai-3 averaged 8.6 ± 3.6 in greenhouse, while in the field, it averaged 21.5 ± 18.0. The susceptible control "Duster" averaged 81.3 ± 18.7 and 85.9 ± 8.3, in greenhouse and field tests, respectively. However, in this study, PSS at 9 DAI after inoculation with fungal spores showed the most differences between the resistant (Sumai 3) and susceptible (AC Nanda) wheat cultivars. The PSS for the resistant control (Sumai 3) is higher than reported in other studies (Oliver et al., 2008; Jin et al., 2013), but it could be due to the *in vitro* screening method using immature spike culture.

The FHB severity determinations using the *in vitro* spike culture method was positively correlated with the field assessments, especially for the values obtained in C2016 (Table 5.1). This correlation is in agreement with the conslusion of Jin et al. (2013) who also reported a moderate to strong positive correlation ($r = 0.73$, $P < 0.001$) between FHB severity observed during green house and field experiments of 363 U.S. winter wheat accessions. This correlation helps validate the utilization of the *in vitro* spike culture technique to evaluate FHB resistance or severity (Sharma et al., 2018).

In summary, the FHB severity determination by two methods, combined with a positive correlation observed between the disease severity ratings in the *in vitro* immature spike culture method in the laboratory to VRI in the Fusarium nursey at Carman Manitoba, support the hypothesis that immature spike culture can be used to screen wheat germplasm

to identify FHB resistant genotypes. The limited FHB nursery results suggest that the *in vitro* spike culture technique can be used used for the evaluation of FHB severity or resistance. The FHB disease severity of SCDV lines at 7 and 11 DAI positively correlated with disease severity rating in the FHB nursery at Carman, Manitoba.

8.1.2 In *in vitro* immature spikes, mycotoxin production in grain is correlated to FHB severity.

Fusarium infection produces mycotoxins in the tricothecene group that, (i) act as virulence factors for the pathogen by inhibiting protein synthesis in plants (Desjardins, 2006; Proctor et al., 2008; Amarasinghe et al., 2015), (ii) are phytotoxic and cause plants to develop non-specific disease symptoms such as chlorosis, necrosis, and wilting (Desjardins, 2006; Nishiuchi et al., 2006), and (iii) in humans negatively affect the integrity, and immunity of human gastrointestinal tract and reduce the absorption of nutrients from food (Liew and Mohd-Redzwan, 2018).

The type B trichothecene mycotoxins deoxynivalenol (DON), nivalenol (NIV) and their acetylated products are the major mycotoxins present in Fusarium infected wheat spikes (Mallmann et al., 2017). Correlation between mycotoxin accumulation and FHB severity is still debated as no (Ji et al., 2015), and weak to moderate ($r = 0.315$-0.610; Miedaner et al., 2016; Huang et al., 2018) positive correlations have been reported in previous studies using liquid chromatography with mass spectrometry, Enzyme-Linked Immunoassay (ELISA) assay, and gas chromatography with mass spectrometry, respectively. Similarly, the association of D3G to DON ratio to FHB resistance is not well established. Nakagawa et al. (2017) found no relationship between D3G to DON ratio and FHB resistance however, the ratio was lower in FHB susceptible genotypes compared to moderately FHB resistant spring wheat varieties (Amarasinghe et al., 2016). To address the second hypothesis, a LC-MS/MS method for simultaneous detection and quantification of multiple type B trichothecenes was developed to determine mycotoxin concentrations in wheat spike samples.

The method developed in this study, separated all the mycotoxins within 15 min. The R^2 values of >0.99 for all the compounds, lower LOD/LOQ values, >80% recovery percentage, and higher repeatability/intermediate precision confirmed the linearity,

sensitivity, accuracy and precision of the optimized method. The compounds in the wheat matrix using the optimized method showed similar SSE patterns therefore, had the least matrix effect on mycotoxins and accounted for >80% recovery of mycotoxins. The modified extraction method omitted the purification and drying steps without compromising mycotoxin separation in LC-MS/MS therefore, it can be characterized as a rapid, easy and economic method. The optimized method used only 0.6 g of ground grain sample to analyze mycotoxin concentrations which is significantly lower compared to other reported methods (5.0-25.0 g) (Kokkonen and Jestoi, 2009; Yoshinari et al., 2012; Zuo et al., 2018) (Table 4.1).

The FHB -resistant and -susceptible genotypes showed distinct accumulation of DON, D3G and 3-ADON at all the stages after fungal inoculation. FHB severity varied significantly between FHB -resistant and -susceptible genotypes at 7 and 9 DAI. FHB infection initiated around 2 DAI, but at 5 DAI, the FHB severity between the susceptible and resistant genotypes could be visibly distinguished but the difference was not statistically significant due to very high standard deviations in the susceptible genotypes. The linear association of FHB disease development to mycotoxin accumulation in wheat spikes concurred with previous studies that also showed a significant positive correlation between mycotoxin (DON and 3-ADON) concentrations and FHB severity in wheat (Tunali et al., 2006; Nobili et al., 2011; Palazzini et al., 2015; Sharma et al., 2018) and other small grain cereals (Wegulo, 2012). Free mycotoxins such as DON and 3-ADON are considered a virulence factor for Fusarium therefore, required for FHB incidence, disease development and severity (Gunupuru et al., 2017). DON is the major mycotoxin produced by the *F. graminearum* isolate M7-07-1 followed by 3-ADON whereas D3G is the product of DON glucosylated by *UDP-Glucosyl Transferase(s)* (Sharma et al., 2018). The conversion of DON to D3G in plants reduces DON accumulation thus provides tolerance/resistance against FHB infection (Lemmens et al., 2005; Sharma et al., 2018). Therefore, a higher D3G:DON ratio was observed at 7 and 9 DAI in FHB resistant genotypes (0.51-0.83 and 0.75-1.00 with averages of 0.63 and 0.85) compared to the susceptible genotypes (0.27-0.43 and 0.57-0.66 with averages of 0.33 and 0.61), respectively which led to a negative correlation between D3G:DON ratio and FHB severity agreeing with earlier reports (Ovando-Martínez et al., 2013). Besides DON, genetic,

environmental and fungal chemotype also influence DON concentration and FHB incidence/severity/fungal biomass (Wegulo, 2012). In Chapter 6, five different *F. graminearum* isolates (M9-07-1, M1-07-2, Canrman-NIV, China-Fg809, and Carman-705-2) grouped in to two categories: (1) nivalenol producing chemotype consisting only Carman-NIV, and (2) the DON and derivative producing chemotypes which included M9-07-1, M1-07-2, China-Fg809, and Carman-705-2 (Figure 6.1). LC-MS/MS was used to determine the mycotoxin concentration which showed positive correlation with FHB severity (Table 6.1). In conclusion, results from infection with single or multiple Fusarium isolates and determination of multiple toxins by LC-MS/MS support the hypothesis that in immature spike cultures, the mycotoxin concentration is positively correlated to FHB severity and disease progression.

8.1.3 Development of DNA based molecular markers for FHB resistance

FHB resistance in wheat is a quantitative trait (Snijders, 2004) with more than 200 QTL having been reported (Jia et al., 2018). The QTL, *Qfhs.ndsu-3BS* or *Fhb1* is the most repeatable (Buerstmayr et al., 2009; Liu et al., 2006; Waldron et al., 1999) and genotypes carrying it exhibit a greater deoxynivalenol-3-O-glucoside:deoxynivalenol (D3G:DON) ratio than lines without *Fhb1* (Rawat et al., 2016), implying that the metabolism of DON plays a significant role in FHB resistance (Lemmens et al., 2005). The detoxification of DON to D3G was associated with *UDP-glucosyltransferase* (*UGT*) genes as they were present on the same chromosome (Rawat et al., 2016). The members of the *UDP-glucosyltransferase* (*UGT*) gene family encode *UDP-glucosyltransferases* (EC 2.4.1.x) that catalyze the transfer of glucose from UDP-glucose to the hydroxyl group at carbon-3 of deoxynivalenol leading to production of non-toxic DON-3-O-glucoside (D3G) (Wetterhorn et al., 2016). Recently SCDV FHB -resistant and -susceptible wheat genotypes were used to confirm the association of *TaUGT-2B* and *TaUGT-3B* with FHB disease development and DON accumulation in grains (Sharma et al., 2018). Further, the SNP in *TaUGT-2B* and *TaUGT-3B* were associated with FHB resistance in wheat. The two SNP identified, at position 450 bp of *TaUGT-2B* and the other at position of 1558 bp respectively from the initiation site, significantly ($P<0.01$) correlated to FHB severity ratings. A HRM assay was used to identify SNP in *TaUGT-3B* in SCDV genotypes and the progeny derived

from several cross combinations between wheat PT588 and SCDV FHB lines (Figure 7.1; 7.2). Four SCDV lines FHB202, FHB244, FHB250 and FHB256 were used as resistant parents BC_2 progenies derived from breeding populations were generated from eight crossing combinations FHB202/PT588, FHB244/PT588, FHB250/PT588, FHB256/PT588, PT588^{*2}/FHB202, PT588^{*2}/FHB244, PT588^{*2}/FHB250, PT588^{*2}/FHB256. Genotyping analysis by HRM showed that the resistant progenies with the same SNP as Sumai-3 accounted for 5.88%, 9.09%, 34.62%, 10.53%, 0.0%, 0.0%, 17.39%, and 27.27% in each F_2 population. The phenotypic results using the immature spike culture assay, the FHB disease severity correlated well with the genotypic analysis. The ANOVA results of FHB severity showed clear separation of FHB severity between resistant and susceptible genotypes ($P<0.01$). In conclusion, the results supported the hypothesis molecular markers can be used to track FHB in a crop improvement program.

8.2 Novel findings

1. An immature spike method was optimized to screen for FHB resistance in wheat.

2. A SNP marker *TaUGT-3B* was optimized in a HRM assay to identify wheat genotypes carrying *Fhb1*.

3. A LC-MS/MS method was optimized to simultaneously detect and quantify four mycotoxin (DON, 3-ADON, 15-ADON, NIV) and D-3-G in wheat spikes.

4. In immature wheat spikes, for DON, five wheat genotypes have been identified with resistance to multiple Fusarium isolates by immature spike culture screening.

5. Markers developed in this study can be used in tracking FHB resistance in wheat progenies between resistant genotypes and and an elite common spring wheat cultivar.

8.3 Future research directions

This study has identified new wheat genotypes with FHB resistance by *in vitro* spike culture, LC-ESI-MS/MS, and HRM analysis. Most of the identified and characterized *Fhb* QTL explain very limited phenotypic variance in FHB resistance, therefore there is an urgent need for new genetics that can be used to develop durable FHB resistance in wheat. The identified genotypes can also be used to identify novel genes related to FHB resistance and elucidate the mechanism underlying FHB resistance.

1. RNA Sequencing analysis between contrasting FHB resistant genotypes can be used to identify additional candidate genes associated with FHB resistance.

2. The validity of candidate genes can be tested by complementation assay.

3. To conduct field evaluations of FHB resistance of selected wheat SCDV genotypes. The determine grain mycotoxin concentrations under field conditions.

4. To cross the selected wheat resistant genotypes with locally adapted wheat genotypes, to develop FHB resistant cultivars.

5. To develop rapid methods to simultaneously detect multiple mycotoixn with more precision, and sensitivity.

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